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(54) Title: BETA-SECRETASE TRANSGENIC ORGANISMS, ANTI-BETA-SECRETASE ANTIBODIES, AND METHODS OF  
USE THEREOF

(57) Abstract: Transgenic non-human animals, including, for example, transgenic rodents and transgenic non-human mammalian  
cells, which harbor a transgene that eliminates the expression of the  $\beta$ -secretase, BACE1, are provided. In addition, antibodies spe-  
cific for BACE1 are provided. Also provided are methods of diagnosing a neurodegenerative disease, including Alzheimer's disease,  
and methods of identifying agents that modulate or treat Alzheimer's disease, and methods of identifying agents that modulate or  
treat Alzheimer's disease and related pathology.



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**BETA-SECRETASE TRANSGENIC ORGANISMS,  
ANTI-BETA-SECRETASE ANTIBODIES,  
AND METHODS OF USE THEREOF**

**BACKGROUND OF THE INVENTION**

**FIELD OF THE INVENTION**

The present invention relates generally to neurological diseases, and more specifically to transgenic non-human organism in which the beta-secretase, BACE1, gene is disrupted and which provide a model system for Alzheimer's disease and related disorders, to antibodies specific for BACE1, and to methods of using such transgenic organisms and antibodies.

**BACKGROUND INFORMATION**

The amyloidoses are a group of pathological conditions in which normally soluble proteins polymerize to form insoluble amyloid fibrils and amyloid deposits. More than 15 proteins form amyloid fibrils, resulting in diverse clinical conditions.

Amyloidoses are usually classified into systemic amyloidoses and localized amyloidoses. Major systemic amyloidoses include AL amyloidosis, amyloid A amyloidosis, and familial transthyretin amyloidosis; the corresponding amyloid proteins associated with these amyloidoses are AL amyloid, amyloid A protein, and transthyretin, respectively. Prominent localized amyloidoses include Alzheimer's disease, prion diseases, and type II diabetes; the corresponding amyloid proteins in these diseases are amyloid  $\beta$  peptide, scrapie prion protein, and human amylin, respectively (Sipe, Ann. Rev. Biochem. 61:947-975, 1992).

Amyloid fibrils, regardless of the amyloid protein from which they are formed, have a cytotoxic effect on various cell types including primary cultured hippocampal neurons (Yankner et al., Science 250:279-282, 1990), pancreatic islet  $\beta$  cells (Lorenzo et al. Nature 368:756-760, 1994) and clonal cell lines (Behl et al., Biochem Biophys. Res. Commun. 186:944-952, 1992; O'Brien et al., Am. J. Pathol. 147:609-616, 1995). In fact, only amyloid proteins in fibrillar form are cytotoxic (Pike et al., Brain Res. 563:311-314, 1991; Lorenzo and Yankner, Proc. Natl. Acad. Sci. 91:12243-12247, 1994). It is likely that the cytotoxic effect of fibrils is mediated by a common mechanism (Lorenzo and Yankner, *supra*; Schubert et al., Proc. Natl. Acad. Sci., USA

92:1989-1993, 1995). Modulation of amyloid protein aggregation is one means of blocking or reducing amyloid toxicity.

Alzheimer's disease (AD) is a progressive disease known generally as senile dementia. The disease falls into two broad categories - late onset and early onset. Late onset AD, which occurs in old age (65+ years), may be caused by the natural atrophy of the brain occurring at a faster rate and to a more severe degree than normal. Early onset AD is much more infrequent, but shows a pathologically identical dementia with brain atrophy that develops well before the senile period, *e.g.*, between the ages of 35 and 60 years.

Alzheimer's disease is characterized by the presence of numerous amyloid plaques and neurofibrillary tangles (highly insoluble protein aggregates) present in the brains of AD patients, particularly in those regions involved with memory and cognition. The production of  $\beta$ -amyloid peptide, a major constituent of the amyloid plaque, can result due to mutations in the gene encoding amyloid precursor protein, which, when normally processed, will not produce the  $\beta$ -amyloid peptide. It is presently believed that a normal (non-pathogenic) processing of the  $\beta$ -amyloid precursor protein occurs via cleavage by a putative " $\alpha$ -secretase," which cleaves between amino acids 16 and 17 of the protein. It is further believed that pathogenic processing occurs via a putative " $\beta$ -secretase" at the amino-terminus of the  $\beta$ -amyloid peptide within the precursor protein. Moreover,  $\beta$ -amyloid peptide appears to be toxic to brain neurons, and neuronal cell death is associated with the disease.

$\beta$ -amyloid peptide (also referred to as A4,  $\beta$ AP, A $\beta$ , or A $\beta$ P; see, U.S. Pat. No. 4,666,829 and Glenner and Wong (1984) *Biochem. Biophys. Res. Commun.* 120:1131) is derived from  $\beta$ -amyloid precursor protein ( $\beta$ APP), which is expressed in differently spliced forms of 695, 751, and 770 amino acids (see, Kang et al., *Nature* 325:773, 1987; Ponte et al., *Nature* 331:525, 1988; and Kitaguchi et al., *Nature* 331:530, 1988). Normal processing of amyloid precursor protein (APP) involves proteolytic cleavage at a site between residues Lys<sup>16</sup> and Leu<sup>17</sup> (as numbered where Asp<sup>597</sup> is residue 1, in Kang et al., *supra*, 1997), near the transmembrane domain,

resulting in the constitutive secretion of an extracellular domain, which retains the remaining portion of the  $\beta$ -amyloid peptide sequence (Esch et al., Science 248:1122-1124, 1990). This pathway appears to be widely conserved among species and present in many cell types (see, Weidemann et al., Cell 57:115-126, 1989; and Oltersdorf et al., J. Biol. Chem. 265:4492-4497, 1990). This normal pathway cleaves within the region of the precursor protein which corresponds to the  $\beta$ -amyloid peptide, thus apparently precluding its formation. Another constitutively secreted form of  $\beta$ APP has been noted (Robakis et al., Soc. Neurosci., Oct. 26, 1993, Abstract No. 15.4, Anaheim, Calif.), which contains more of the  $\beta$ APP sequence carboxy terminal to the form described by Esch et al. (*supra*, 1990).

Soluble  $\beta$ -amyloid peptide is produced by healthy cells into culture media (Haass et al., Nature 359:322-325, 1992) and in human and animal CSF (Seubert et al., Nature 359:325-327, 1992). Palmert et al. (Biochem. Biophys. Res. Comm. 165:182-188, 1989) describe three possible cleavage mechanisms for  $\beta$ APP and present evidence that  $\beta$ APP cleavage does not occur at methionine<sup>596</sup> in the production of soluble derivatives of  $\beta$ APP. U.S. Pat. No. 5,200,339, describes the existence of certain proteolytic factor(s), which putatively are capable of cleaving  $\beta$ APP at a site near the  $\beta$ APP amino-terminus.

The *APP* gene is located on human chromosome 21. A locus segregating with familial Alzheimer's disease has been mapped to chromosome 21 (St. George Hyslop et al., Science 235:885, 1987) close to the *APP* gene. Recombinants between the *APP* gene and the AD locus have been reported (Schellenberg et al., Science 241:1507, 1988; Schellenberg et al., Am. J. Hum. Genetics 48:563, 1991; Schellenberg et al., Am. J. Hum. Genetics 49:511, 1991).

The identification of mutations in the amyloid precursor protein gene that cause familial, early onset Alzheimer's disease is evidence that amyloid metabolism is the central event in the pathogenic process underlying the disease. Four reported disease-causing mutations include, with respect to the 770 isoform, V717I (Goate et al., Nature 349:704, 1991), V717G (Chartier Harlan et al., Nature 353: 844, 1991),

V717F (Murrell et al., Science 254:97, 1991), and with respect to the 695 isoform, a double mutation changing K595N and M596L (Mullan et al., Nature Genet 1:345, 1992; Citron et al., Nature 360:672, 1992) referred to as the Swedish mutation.

The development of experimental models of AD that can be used to further study the underlying biochemical events involved in AD pathogenesis would be highly desirable. Such models could presumably be employed, in one application, to screen for agents that alter the degenerative course of Alzheimer's disease. For example, a model system of AD can be used to screen for environmental factors that induce or accelerate the pathogenesis of AD. An experimental model also can be used to screen for agents that inhibit, prevent, or reverse the progression of AD. Presumably, such models could be employed to develop pharmaceuticals that are effective in preventing, arresting, or reversing AD. It would also be desirable to have a model that can be used as a standard or control for comparison of agents the modulate amyloid deposition or activity. Thus, a need exists for a model system of Alzheimer's disease. The present invention satisfies this need, and provides additional advantages.

### **SUMMARY OF THE INVENTION**

The present invention provides a method for modulating the production of A $\beta$ 11-40/42 peptide fragments. The method includes contacting a sample or cell containing a beta-site APP-cleaving enzyme 1 (BACE1) and an amyloid precursor protein (APP) with a BACE1-modulating agent such that production of A $\beta$ 11-40/42 is modulated. The contacting can be *in vivo* or *in vitro*.

In another embodiment, the invention provides a method for identifying a compound that inhibits BACE1 expression or activity. The method includes incubating components including the compound, BACE1 polynucleotide or polypeptide, and an APP under conditions sufficient to allow the components to interact, and measuring the production of a BACE1 specific enzymatic product.

Also provided are methods for diagnosing a subject having or at risk of having an A $\beta$ 11-40/42 peptide accumulation disease. The method includes measuring the

amount of BACE1 in a biological sample from the subject; and comparing the amount BACE1 with a normal standard value of BACE1, wherein a difference between the measured amount and the normal sample or standard value provides an indication of the diagnosis of A $\beta$ 11-40/42. The sample can be, for example, blood, serum, cerebrospinal fluid or central nervous system (CNS) tissue.

In yet another embodiment, the invention provides a method for diagnosing a subject having or at risk of having Alzheimer's disease, including measuring A $\beta$ 11-40/42 in a biological sample from the subject; comparing the amount of A $\beta$ 11-40/42 with a normal sample or standard value of A $\beta$ 11-40/42, wherein a difference between the amount in the normal sample or standard value is indicative of a subject having or at risk of having Alzheimer's disease.

In another embodiment, the invention provides a transgenic non-human animal having a transgene disrupting expression of BACE1, chromosomally integrated into the germ cells of the animal, and have a phenotype of reduced A $\beta$  peptide as compared with a wild-type animal. The invention also provides a method for producing a transgenic non-human animal having a phenotype characterized by reduced expression of BACE1 polypeptide. The method includes introducing at least one transgene into a zygote of an animal, the transgene(s) comprising a DNA construct encoding a selectable marker, transplanting the zygote into a pseudopregnant animal, allowing the zygote to develop to term, and identifying at least one transgenic offspring whose genome comprises a disruption of the endogenous BACE1 polynucleotide sequence by the transgene.

In yet another embodiment, the invention provides a method for identifying an agent that modulates the expression or activity of BACE1. The method includes administering an agent to be tested to an organism; and comparing the phenotype of the organism contacted with the agent with that of a BACE1 knockout organism not contacted with the agent, whereby a phenotype substantially equal to the BACE1 knockout organism is indicative of an agent that modulates BACE1 expression or activity.

The invention also provides a method for screening for an agent, which ameliorates symptoms of Alzheimer's disease. The method includes comparing an effect of an agent on an organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent, wherein the organism has a phenotype associated with Alzheimer's disease and wherein an agent which ameliorates said phenotype is identified by having a substantially equal or superior phenotype of the organism in comparison with the BACE1 knockout organism.

In yet another embodiment, the invention provides a method for screening for an agent, which ameliorates symptoms of Alzheimer's disease. The method includes comparing an effect of an agent on a transgenic organism contacted with the agent with that of a BACE1 knockout organism not contacted with the agent, wherein the transgenic organism is characterized as having a phenotype of impaired performance on memory learning tests or abnormal neuropathology in a cortico-limbic region of the brain and the BACE1 knockout organism has a phenotype of reduced expression of BACE1, wherein the impaired performance and the abnormal neuropathology are in compared with the BACE1 knockout organism, whereby an agent which ameliorates the symptoms is identified by substantially equal or superior performance of the transgenic organism as compared with the BACE1 knockout organism on the memory and learning tests.

The invention also provides a kit useful for the detection of an A $\beta$ 11-40/42 accumulation disorder comprising carrier means containing therein one or more containers wherein a first container contains a nucleic acid probe that hybridizes to a nucleic acid sequence BACE1 or an antibody probe specific for BACE1 or A $\beta$ 11-40/42.

In yet another embodiment, the invention provides a method for predicting the therapeutic effectiveness of a compound for treating Alzheimer's disease in a subject by measuring the accumulation of A $\beta$ 11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide before and after treatment with the

compound, wherein a decrease in accumulation of peptide fragments or a decrease in the level of BACE1 polynucleotide or polypeptide after treatment is indicative of a compound that is effective in treating the disease.

In another embodiment, the invention provides a method for monitoring the progression of Alzheimer's disease by measuring the accumulation of A $\beta$ 11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide at a first time point and a second time point, thereby monitoring the progression of the disease.

The invention also provides antibodies that specifically bind BACE1 or a peptide portion of BACE1 corresponding to amino acid residues 46 to 163. The antibodies can be polyclonal antibodies or monoclonal antibodies derived therefrom, and can be in a substantially purified form or can be a component of an antiserum. Also provided are methods of using such anti-BACE1 antibodies to detect BACE1 in a sample, for example, a tissue sample such as a brain tissue sample, or to diagnose a disorder associated with an accumulation of amyloid plaques, for example, Alzheimer's disease. As disclosed herein, BACE1 has the characteristics of a susceptibility factor that contributes to brain-specific A $\beta$  amyloidogenesis. BACE1 was found to be abundant in the brain, and is particularly rich in the hippocampus, including the giant boutons of hippocampal mossy fibers, a region that is critical for learning and memory and especially vulnerable in AD. Whereas high levels of BACE1 coupled with low levels of  $\alpha$ -secretase and BACE2 activities were observed in neurons, low levels of BACE1 and high levels of BACE2 and  $\alpha$ -secretase activities were detectable in non-neuronal cells. Importantly, while the deletion of BACE1 abolished the secretion and deposition of A $\beta$ , the partial reduction of BACE1 (to 50% of normal level) significantly ameliorated amyloid plaque deposition in a mouse model of A $\beta$  amyloidosis. These results demonstrate that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of A $\beta$  in the central nervous system and indicate that polymorphisms that increase levels of BACE1 can have a role in predisposing an individual to AD. Accordingly, the present invention further relates to methods of determining whether an individual has, or is susceptible

or predisposed to developing, A $\beta$  deposition in the brain, including to an amyloidosis such as AD, and further relates to methods of modulating BACE1 activity in a cell, and to methods of preventing or of ameliorating A $\beta$  deposition in an individual.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1A is a map of the wild-type *BACE1* locus, the targeting vector, and the disrupted *BACE1* allele. The first coding exon of BACE1 is indicated by black box. The targeting vector shows the replacement of the first coding exon and flanking genomic sequences by the neomycin gene (*neo*) and the HSV thymidine kinase gene (*tk*). Arrows indicate the sites within the targeted and wild-type alleles from which PCR primers were chosen for genotyping. Lines below denote expected sizes for *SacI*-digested fragments detected by a 5'-flanking probe (a 0.45 kb *HindIII/PstI* fragment, black bar) from targeted and endogenous *BACE1* alleles. B, *BamHI*; H, *HindIII*; P, *PstI*; S, *SacI*; X, *XbaI*.

Figure 1B shows an analysis of genomic DNA from *BACE1*<sup>+/-</sup> crosses by Southern blot. The *SacI* fragments detected for wild-type (8.0 kb) and targeted (5.4 kb) *BACE1* alleles with the 5' probe are indicated.

Figure 1C shows PCR analysis of DNA extracted from embryos using primers indicated in A, the 157 bp or 272 bp fragment is specific to the targeted or endogenous *BACE1* allele, respectively.

Figure 1D shows total protein extracts (30  $\mu$ g) of wild-type (+/+), heterozygous (+/-), and homozygous BACE1 knockout (-/-) from E16.5 embryos. Embryos were immunoblotted using rabbit polyclonal antisera specific for epitopes in the N-terminal 46-163 amino acids of BACE1, and superoxide dismutase 1 (SOD1).

Figure 2A shows a sequence alignment of A $\beta$ 1-42 denoting differences between the human and mouse protein sequences (bolded amino acids). The cleavage sites corresponding to BACE1,  $\alpha$  and  $\gamma$  secretases are marked and numbered. The asterisk indicate the start of the transmembrane domain.

Figure 2B shows IP-MS analysis of secreted A $\beta$  peptides from primary cultured cortical neurons derived from wide-type (+/+), heterozygous (+/-), and homozygous BACE1 knockout (-/-) E16.5 embryos using the Ciphergen ProteinChip™ system. Peaks corresponding to mouse A $\beta$  peptides, 17-40, 11-40, 11-42, 1-40 and 1-42 are marked by asterisk. The mass of each peptide is labeled within brackets.

Figure 2C shows a determination of A $\beta$ 1-40 and A $\beta$ 1-42 levels from conditioned media of BACE1<sup>+/+</sup> and BACE1<sup>-/-</sup> neuronal cultures following 4 days of infection with adenovirus expressing humanized APPswe by ELISA. The concentrations of A $\beta$  peptides for each genotype are plotted (pg/ml) as mean +/- standard deviation (n=3).

Figure 2D shows conditioned media from BACE1<sup>+/+</sup> and BACE1<sup>-/-</sup> neuronal cell cultures radiolabeled with <sup>35</sup>S-methionine after 4 days of infection with recombinant adenovirus expressing humanized APPswe were immunoprecipitated with 4G8, an antisera specific for A $\beta$  peptides.

Figure 2E shows a detergent lysates from BACE1<sup>+/+</sup> and BACE1<sup>-/-</sup> neuronal cell cultures radiolabeled with <sup>35</sup>S-methionine after 4 days of infection with recombinant adenovirus expressing humanized APPswe. The cells were immunoprecipitated with CT15, an antisera recognizing APP C terminus. BACE1 deficient neurons failed to generate APP  $\beta$ -CTF.

Figures 3A-3D show a gel from neuronal cultures infected with adenovirus. Following 4 days of infection with adenovirus expressing humanized APPswe, BACE1<sup>+/+</sup> (Figure 3A; lanes 1-4) and BACE1<sup>-/-</sup> (lanes 5-8) neuronal cultures were pulse-labeled for 45 minutes (lanes 1 and 5) with <sup>35</sup>S-methionine, then chased in the presence of cold L-methionine for 1 hr (lanes 2 and 6), 2 hr (lanes 3 and 7), and 4 hr (lanes 4 and 8). Full-length APP and CTFs of APP were immunoprecipitated with CT15. A $\beta$  and p3 peptides (Figure 3B), soluble APP derivatives (APP<sup>s</sup>) (Figure 3C), or  $\alpha$ -secretase-generated APP<sup>s</sup> (APP<sup>s</sup> $\alpha$ ; Figure 3D), were immunoprecipitated with

4G8, 22C11, or 6E10 antisera, respectively, from conditioned media of the corresponding neuronal cultures (Figure 3A).

Figure 3E is a quantitative analysis of APP<sup>sα</sup> release. Experiments were performed in duplicate on different days. The APP<sup>sα</sup> and APP<sup>s</sup> signals at each point of the pulse-chase experiments were quantified by phospho-imaging.

Figure 4 shows that cultured astrocytes have greater BACE2 and α-secretase activity as compared to neurons. The signal intensities of Aβ1-15, Aβ1-6, Aβ1-19, and Aβ1-20 in glia and astrocytes are shown normalized with respect to Aβ1-40 (see Example 7).

#### **DETAILED DESCRIPTION OF THE INVENTION**

Alzheimer's disease (AD) is a progressive neurodegenerative disorder causing dementia in the elderly that is characterized, in part, by the deposition of Aβ-amyloid and by neurofibrillary tangles in a variety of brain region, particularly the hippocampus and cerebral cortex. Endoproteolytic cleavages of APP by α-secretase and β-secretase activities result in the generation of toxic Aβ peptides. Two homologous β-secretases, termed BACE1 and BACE2, have been cloned and shown to be transmembrane aspartyl proteases that cleave APP at the +1 Aβ site. Initial studies indicated that *BACE1* and *BACE2* mRNA are expressed ubiquitously, although BACE2 is expressed at lower levels in brain.

The present invention is based upon the discovery that BACE1-knockout transgenic organisms lacking normal expression of BACE1 have reduced accumulation of APP peptide fragments. The transgenic organisms have led to the discovery that BACE1 is the β-secretase responsible for the Aβ<sub>1-42</sub> peptide fragment of APP. Accordingly, the invention provides diagnostic methods and compositions useful for detecting AD as well as other BACE1-associated and APP-associated disorders. Based on the discovery of the role of BACE1 in AD, the invention also provides screening assays useful for identifying drugs that can inhibit or prevent Aβ<sub>1-42</sub> production and, therefore, may be effective for AD treatment.

The term "isolated" or "substantially purified" means altered "by the hand of man" from its natural state; *i.e.*, if a material occurs in nature, reference to the material as being "isolated" means that it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated," as the term is employed herein. Thus, anti-BACE1 an antiserum, which contains anti-BACE1 antibodies and is obtained from an immunized animal such as a rabbit, is removed from its natural state, *i.e.*, the rabbit's circulatory system, and, therefore, is an example of a substantially purified material.

As part of or following isolation, a polynucleotide can be joined to other polynucleotides, such as heterologous DNA molecules, for mutagenesis studies, to form fusion proteins, and for propagation or expression of the polynucleotide in a host. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells in culture or in whole organisms. Such polynucleotides, when introduced into host cells in culture or in whole organisms, still are considered isolated as the term is used herein because they are not in their naturally occurring form or environment. Similarly, the polynucleotides and polypeptides can be present in a composition such as a medium formulation (solutions for introduction of polynucleotides or polypeptides, for example, into cells or compositions or solutions for chemical or enzymatic reactions).

Polynucleotide or nucleic acid sequence refers to a polymeric form of nucleotides. In some instances a polynucleotide refers to a sequence that is not immediately contiguous with either of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, the term "isolated" includes, for example, a recombinant DNA molecule, which can be incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which can exist as a separate molecule (*e.g.*, a cDNA) independent of other sequences. The

nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. In addition, the polynucleotide sequence involved in producing a polypeptide chain can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons) depending upon the source of the polynucleotide sequence.

The term "polynucleotide" generally refers to any polyribonucleotide or polydeoxyribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. Thus, the term "polynucleotides" includes, for example, single stranded and double stranded DNA, DNA that contains both single stranded and double stranded regions, single stranded and double stranded RNA, and RNA that contains both single stranded and double stranded regions, hybrid molecules comprising DNA and RNA that may be single stranded or, more typically, double stranded or a mixture of single stranded and double stranded regions. In addition, a polynucleotide can contain triple stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions can be from the same molecule or from different molecules. The regions can include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple helical region often is an oligonucleotide.

A polynucleotide or nucleic acid sequence can contain one or more modified bases. Thus, DNA or RNA molecules with backbones modified for stability or for other reasons are considered "polynucleotides" as the term is used herein. Moreover, DNA or RNA molecules comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are considered polynucleotides as the term is used herein.

Polynucleotides can be created that encode a fusion protein and can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a coding sequence is "operatively linked" to another coding sequence when RNA polymerase will transcribe the two coding

sequences into a single mRNA, which can be translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein. An expression control sequence operatively linked to a coding sequence is positioned such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences.

As used herein, the term "expression control sequence" or "control sequences" refers to a nucleotide sequence that regulates the expression of a polynucleotide to which it is operatively linked. Expression control sequences are operatively linked to a polynucleotide when the expression control sequences control and regulate the transcription and, as appropriate, translation of the polynucleotide. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) at the beginning of a protein-encoding sequence, splice signals for introns, which allow for maintenance of the correct reading frame of a polynucleotide containing introns and, therefore, translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, elements whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

Expression control sequences include promoters, enhancers, silencers, and the like. The term "promoter" is used to refer to a minimal sequence sufficient to direct transcription. Also included are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements generally are positioned 5' to the coding sequence, though enhancer elements also can have promoter activity, in which case the element can be positioned 3' to a coding region of a polynucleotide. Constitutive and inducible promoters are useful for purposes of the present invention (see e.g., Bitter et al., *Methods in Enzymology* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as *pL* of bacteriophage, *plac*, *ptrp*, *ptac* (*ptrp-lac* hybrid promoter) and the like may be used. When cloning in mammalian cell

systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) can be used. Promoters produced by recombinant DNA or synthetic techniques also can be used to provide for transcription of the nucleic acid sequences of the invention.

A polynucleotide of the invention including, for example, a polynucleotide encoding a fusion protein, can be inserted into a recombinant expression vector. A recombinant expression vector generally is a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of a desired nucleotide sequences. For example, a recombinant expression vector of the invention includes a polynucleotide sequence encoding a polypeptide having BACE1 activity or a fragment thereof, or encoding an APP fusion product or fragment thereof. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al., *Gene* 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.* 263:3521, 1988), baculovirus-derived vectors for expression in insect cells, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV.

A polynucleotide of the invention can also can be operatively linked to a localization sequence such as a nuclear localization signal, signal peptide, or the like, which can direct the linked molecule to particular cellular sites by fusion to appropriate organellar targeting signals or localized host proteins. For example, a polynucleotide encoding a localization sequence, or signal sequence, can be used as a repressor and thus can be operatively linked at the 5' terminus of a polynucleotide encoding a polypeptide of the invention such that the localization or signal peptide is located at the amino terminal end of a resulting polynucleotide/polypeptide. The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor,

NY, 1989, and Current Protocols in Molecular Biology, M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., most recent Supplement)). These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination.

In yeast, a number of vectors containing constitutive or inducible promoters can be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel, et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Grant, et al., "Expression and Secretion Vectors for Yeast," in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp.516-544, 1987; Glover, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; and Bitter, "Heterologous Gene Expression in Yeast," Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684, 1987; and The Molecular Biology of the Yeast *Saccharomyces*, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II, 1982. A constitutive yeast promoter such as *ADH* or *LEU2* or an inducible promoter such as *GAL* can be used ("Cloning in Yeast," Ch. 3, R. Rothstein In: DNA Cloning Vol.11, A Practical Approach, Ed. DM Glover, IRL Press, Wash., D.C., 1986). Alternatively, vectors can be used which promote integration of foreign DNA sequences into the yeast chromosome.

An alternative expression system which could be used to express a BACE (e.g., BACE1) polypeptide of the invention is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign or mutated polynucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The sequence encoding a protein of the invention may be cloned into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an *AcNPV* promoter (for example the polyhedrin promoter). Successful insertion of the sequences coding for a protein of the invention will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed (see Smith et al., J. Virol. 46:584, 1983; Smith, U.S. Pat. No. 4,215,051).

A polynucleotide of the invention, which can be contained in a vector, can be used to transform a host cell. By transform or transformation is meant a permanent or transient genetic change induced in a cell following incorporation of new DNA (*i.e.*, DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. A transformed cell or host cell generally refers to a cell (*e.g.*, prokaryotic or eukaryotic) into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding an APP or BACE polypeptide or a fragment thereof.

Transformation of a host cell with recombinant DNA can be carried out by conventional techniques as are well known to those skilled in the art. Where the host is a prokaryotic cell such as *E. coli*, competent cells that are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the  $\text{CaCl}_2$  method by procedures well known in the art. Alternatively,  $\text{MgCl}_2$  or  $\text{RbCl}$  can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host cell is a eukaryotic cell, methods of transfection or transformation with DNA include calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors, as well as others known in the art, may be used. Eukaryotic cells can also be cotransfected with DNA sequences encoding a BACE1 polypeptide and a second foreign DNA molecule encoding APP, or a selectable marker, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982). Typically, a eukaryotic host will be utilized as the host cell. The eukaryotic cell may be a yeast cell (*e.g.*, *Saccharomyces cerevisiae*), an insect cell (*e.g.*, *Drosophila sp.*) or may be a mammalian cell, including a human cell.

Eukaryotic systems, including mammalian expression systems, allow for post-translational modifications of expressed mammalian proteins to occur. Eukaryotic cells that possess the cellular machinery for processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used. Such host cell lines may include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, Jurkat, HEK-293, and WI38.

Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression can be engineered. For example, when using adenovirus expression vectors, a polynucleotide encoding a BACE (e.g., BACE1) polypeptide may be ligated to an adenovirus transcription/ translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric sequence may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a BACE polypeptide or a fragment thereof in infected hosts (see Logan and Shenk, Proc. Natl. Acad. Sci. USA, 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter can be used (see Mackett et al., Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett et al., J. Virol. 49:857-864, 1984; Panicali et al., Proc. Natl. Acad. Sci. USA 79:4927-4931, 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the *neo* gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of a BACE gene in host cells (Cone and Mulligan, Proc. Natl. Acad. Sci. USA, 81:6349-6353, 1984). High level expression can also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

For long term, high yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the cDNA encoding an APP, APP fragment or BACE polypeptide controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant vector confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems can be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al., Cell, 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy et al., Cell, 22:817, 1980) genes can be employed in *tk*-, *hgp*rt- or *ap*rt- cells respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler et al., Proc. Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare et al., Proc. Natl. Acad. Sci. USA 8:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan and Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981; *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J. Mol. Biol. 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre *et al.*, Gene 30:147, 1984) genes. Additional selectable markers included *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, Proc. Natl. Acad. Sci. USA 85:8047, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed., 1987).

The term "primer" as used herein refers to an oligonucleotide, whether natural or synthetic, which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated or possible. Synthesis of a primer

extension product that is complementary to a nucleic acid strand is initiated in the presence of nucleoside triphosphates and a polymerase in an appropriate buffer at a suitable temperature. For example, if a polynucleotide sequence is inferred from a polypeptide sequence, a primer generated to synthesize the polynucleotide encoding the polypeptide sequence can be a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One or more of the primers in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved" region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences.

The term "polypeptide" or "protein" refers to a polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being typical. Examples of polypeptides useful in the methods and compositions of the invention include APP (see, for example, Cheler, J. Neurochem. 65(4):1431, 1995, which is incorporated herein by reference), fragments of APP, including A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ 11-40, and A $\beta$ 11-42; and BACE1 (see Vassar et al. Science 286:735, 1999, which is incorporated herein by reference). Accordingly, the polypeptides of the invention are intended to encompass isolated naturally occurring proteins, as well as those generated recombinantly or synthesized using chemical or biological methods.

Polypeptide fragments are also encompassed by the invention. Such fragments can have the same or substantially the same amino acid sequence as a portion of the naturally occurring protein. A polypeptide or peptide having substantially the same sequence means that an amino acid sequence is largely, but not entirely, the same, but retains a functional activity of the sequence to which it is related. In general polypeptides of the invention include peptides, or full length protein, that contains substitutions, deletions, or insertions into the protein backbone, that would still have about 70%, generally about 80%, and particularly about 90% sequence identity to the original (reference) protein over the corresponding portion. A yet greater degree of departure from

homology can be allowed if conservative amino acid substitutions are considered as sharing identity with the substituted amino acid residue.

A polypeptide can be substantially related, but for a conservative variation, such polypeptides being encompassed within the present invention. A conservative variation denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine. A conservative variation also can be due to the use of a substituted amino acid in place of an unsubstituted amino acid, provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, or configuration concerns, one skilled in the art will recognize the need to introduce a modification by deletion, replacement, or addition of one or more amino acid residues. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, cytosine for specific disulfide bridge formation. The modified peptides can be chemically synthesized, or the isolated gene can be site-directed mutagenized, or a synthetic gene can be synthesized and expressed in bacteria, yeast, baculovirus, tissue culture and so on.

The present invention also provides a substantially purified antibody that specifically binds a BACE1 polypeptide or an epitopic determinant thereof, and antigen binding fragments of such antibodies. The antibody can be in the form of an antiserum, which is isolated from an immunized animal, can be in the form of substantially purified polyclonal antibodies, which have been isolated from an antiserum containing anti-BACE1 antibodies, or can be in the form of a monoclonal antibody. The epitopic determinant of BACE1, to which an antibody of the invention specifically binds, can be any portion of BACE1, including a contiguous amino acid sequence or a two or more sequences of the BACE1 polypeptide that are in proximity in the three dimensional structure of BACE1, provided the epitope is unique to BACE1 such that the antibodies of the invention do not substantially cross-react with an unrelated polypeptide. For example, the epitopic determinant can include a structure formed by a peptide containing amino acid residues 46 to 164 of BACE1 (see Example 2).

The term "specifically binds," when used herein in reference to an antibody, means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about  $1 \times 10^{-6}$ , generally at least about  $1 \times 10^{-7}$ , usually at least about  $1 \times 10^{-8}$ , and particularly at least about  $1 \times 10^{-9}$  or  $1 \times 10^{-10}$  or less. As such, Fab, F(ab')<sub>2</sub>, Fd and Fv fragments of an antibody that retain specific binding activity for BACE1 or a BACE1 epitope are included within the definition of an antibody. The term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric antibodies, bifunctional antibodies and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., Science 246:1275-1281, 1989). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246, 1993; Ward et al., Nature 341:544-546, 1989; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988); Hilyard et al., Protein Engineering: A

practical approach (IRL Press 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995)).

Antibodies of the invention can be prepared as disclosed herein or using other methods as are well known and routine in the art. Where a peptide portion of BACE1 used as an immunogen is non-immunogenic, it can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art (see, for example, by Harlow and Lane, *supra*, 1988). Methods for raising polyclonal antibodies, for example, in a rabbit, goat, mouse or other mammal, are well known in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed., Humana Press 1992), pages 1-5; Coligan et al., "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters," in *Curr. Protocols Immunol.* (1992), section 2.4.1).

Monoclonal antibodies also can be obtained using methods that are well known and routine in the art (Kohler and Milstein, *Nature* 256:495, 1975; Coligan et al., *supra*, 1992, sections 2.5.1-2.6.7; Harlow and Lane, *supra*, 1988). For example, spleen cells from a mouse immunized with BACE1, or an epitopic fragment thereof, can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using, for example, labeled BACE1 to identify clones that secrete monoclonal antibodies having the appropriate specificity, and hybridomas expressing antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies. Polyclonal antibodies similarly can be isolated, for example, from serum of an immunized animal. Such antibodies, in addition to being useful for performing a method of the invention, also are useful, for example, for preparing standardized kits. A recombinant phage that expresses, for example, a single chain antibody also provides an antibody that can be used for preparing standardized kits.

Monoclonal antibodies, for example, can be isolated and purified from hybridoma cultures by a variety of well established techniques, including, for example, affinity chromatography with Protein-A SEPHAROSE gel, size exclusion chromatography, and ion exchange chromatography (Barnes et al., in Meth. Mol. Biol. 10:79-104 (Humana Press 1992); Coligan et al., *supra*, 1992, see sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3). Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known. For example, multiplication *in vitro* can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* can be carried out by injecting cell clones into mammals histocompatible with the parent cells, for example, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals can be primed with a hydrocarbon, for example, an oil such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

An antigen binding fragment of an antibody that specifically binds BACE1 also is considered encompassed within the antibodies of the present invention. An antigen binding fragment of an antibody can be used in a method of the invention, as can an antibody derived from such an antibody, for example, a single chain antibody. An antigen binding fragment of an antibody can be prepared by proteolytic hydrolysis of a particular antibody, or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')<sub>2</sub>. This fragment can be further cleaved using a thiol reducing agent,

and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly (see, for example, Goldenberg, U.S. Patent No. 4,036,945 and U.S. Pat. No. 4,331,647; Nisonhoff et al., Arch. Biochem. Biophys. 89:230, 1960; Porter, Biochem. J. 73:119, 1959; Edelman et al., Meth. Enzymol., 1:422 (Academic Press 1967); Coligan et al., *supra*, 1992, see sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light/heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques can also be used, provided the fragments specifically bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of variable heavy ( $V_H$ ) chains and variable light ( $V_L$ ) chains, which can be a noncovalent association (Inbar et al., Proc. Natl. Acad. Sci., USA 69:2659, 1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (Sandhu, Crit. Rev. Biotechnol. 12:437, 1992). Preferably, the Fv fragments comprise  $V_H$  and  $V_L$  chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the  $V_H$  and  $V_L$  domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are well known (see, for example, by Whitlow et al., "Methods: A Companion to Methods in Enzymology" 2:97, 1991; Bird et al., Science 242:423-426, 1988; Ladner et al., U.S. Pat. No. 4,946,778; Pack et al., BioTechnology 11:1271-1277, 1993; Sandhu, *supra*, 1992).

Another example of an antigen binding fragment of an antibody is a peptide coding for a single complementarity determining region (CDR). CDR peptides can be obtained by constructing polynucleotides encoding the CDR of an antibody of

interest. Such polynucleotides can be prepared, for example, using the polymerase chain reaction to synthesize a variable region encoded by RNA obtained from antibody-producing cells (see, for example, Larrick et al., *Methods: A Companion to Methods in Enzymology* 2:106, 1991, which is incorporated herein by reference).

Humanized monoclonal antibodies also can be used in a method or kit of the invention if desired. Humanized monoclonal antibodies can be produced, for example, by transferring nucleotide sequences encoding mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. Methods for cloning murine immunoglobulin variable domains are known (see, for example, Orlandi et al., *Proc. Natl. Acad. Sci., USA* 86:3833, 1989), and for producing humanized monoclonal antibodies are well known (see, for example, Jones et al., *Nature* 321:522, 1986; Riechmann et al., *Nature* 332:323, 1988; Verhoeyen et al., *Science* 239:1534, 1988; Carter et al., *Proc. Natl. Acad. Sci., USA* 89:4285, 1992; Singer et al., *J. Immunol.* 150:2844, 1993; Sandhu, *supra*, 1992).

Antibodies useful in a method of the invention also can be derived from human antibody fragments, which can be isolated, for example, from a combinatorial immunoglobulin library (see, for example, Barbas et al., *Methods: A Companion to Methods in Immunology* 2:119, 1991; Winter et al., *Ann. Rev. Immunol.* 12:433, 1994). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library are commercially available (Stratagene; La Jolla CA). In addition, the antibody can be derived from a human monoclonal antibody, which can be obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge (see, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature* 368:856, 1994; and Taylor et al., *Int. Immunol.* 6:579, 1994; see, also, Abgenix, Inc.; Fremont CA).

The present invention further provides a method of detecting a BACE1 polypeptide in a sample. Such a method can be performed, for example, by contacting the sample

with an antibody or antigen binding fragment thereof of the invention under conditions that allow specific binding of the antibody to BACE1 or an epitopic determinant thereof, and detecting specific binding of the antibody to a component of the sample. An immunoassay of the invention can be adapted to any format as desired, including, for example, an ELISA, RIA, and the like, or an immunohistochemical method. The sample can be any sample in which it is desired to know whether BACE1 is present, including, for example, cells or a cell extract, or a tissue sample, which can be obtained from a subject using biopsy methods or other methods for collecting a biological sample as are known in the art. In one embodiment, the sample is a brain tissue sample, for example, from a subject that has, or is suspected of having, a disorder associated with an accumulation of amyloid plaques, and the method is performed in an immunohistochemical format. As such, a method of the invention can be useful for detecting, for example, the presence or absence of BACE1 in a region of the brain containing amyloid plaques, and, therefore, can be diagnostic of an amyloidosis such as Alzheimer's disease.

For purposes of a detecting method of the invention, the anti-BACE1 antibody can be detectably labeled using any reagent as disclosed herein as useful for labeling a polypeptide or, where appropriate, a polynucleotide, as well as any other label conveniently and routinely used in the art. Alternatively, a separate reagent that specifically binds to an anti-BACE1 antibody can be detectably labeled, and can be used to detect specific binding of the anti-BACE1 antibody to a component of the sample. Such a reagent can be a second antibody, which can specifically bind an immunoglobulin class of which the anti-BACE1 antibody is a member, for example, an IgG, IgM, IgA or the like, or can be a reagent such as Protein A.

If desired, an antibody or antigen binding fragment of the invention can be immobilized to a solid support. The solid support can be any material that is substantially insoluble under the conditions to which a method of the invention will be performed, i.e., under conditions in which immunoassays generally are performed. In addition, a material is selected as a solid support based on its stability to conditions under which an antibody is to be immobilized to the support. Thus, a solid support

can be composed of glass, silicon, gelatin, agarose, a metal, or a synthetic material such as a plastic or other polymer, for example, polystyrene, polydextran, polypropylene, polyvinyl chloride, polyvinylidene fluoride, polyacrylamide, and the like.

Where the solid support has a hydrophobic surface, an antibody can be immobilized to the support simply by contacting the antibody and the surface such that the antibody is immobilized through a hydrophobic interaction with the surface, as is typical for solid phase immunoassays. A solid support also can be modified to contain reactive groups that facilitate binding of an antibody to the support, thereby immobilizing the antibody. Alternatively, or in addition, the antibody can be modified to facilitate immobilization to the support, for example, by modifying the antibody to contain a member of a specific binding pair, wherein the second member of the binding pair is a component of the support. For example, the antibody can be covalently bound, for example, to a magnetic iron oxide bead, which can be modified to contain reactive amine groups or carboxyl groups (Pierce Chemical Co.) or a member of a specific binding pair such as streptavidin (DynaL Biotech), thereby immobilizing the antibody and also providing a convenient means to isolate the antibody, as well as any BACE1 polypeptide specifically bound thereto by contacting the mixture with a magnet (see, for example, Bodinier et al., Nat. Med. 6:707-710, 2000). Accordingly, a method of detecting BACE1 in a sample can further include a step of isolating BACE1 that is specifically bound by the antibody.

Prior to the present disclosure, the role of BACE1 in the processing of APP and fragments thereof was not understood. Accordingly, the present invention provides an understanding of the role of BACE1 in the processing of APP and in AD. Thus, in one embodiment, the invention provides a method for modulating (*e.g.*, inhibiting) the interaction of a BACE1 polypeptide with its substrate APP (either *in vitro* or *in vivo*) by administering to a cell or to a subject an effective amount of a composition that contains a BACE1 polypeptide, or a biologically functional fragment thereof, or an agent such as an antibody, ribozyme, antisense molecule, or double stranded interfering RNA

molecules that interacts with or inhibits expression or the activity of a BACE1 polypeptide.

As used herein, an "effective amount" of a composition containing a BACE1 polypeptide or a BACE1 polypeptide-modulating agent is an amount that can modulate the normal enzymatic activity or interaction of a BACE1 substrate with a BACE1 polypeptide or protein in a subject or cell. A "normal" amount of BACE1 activity can be determined using methods as disclosed herein and statistical analyses as are well known in the art.

The present invention also provides a method for modulating expression of a BACE1 polypeptide, as well as methods for screening for agents that modulate BACE1 polypeptide gene expression. According to such a method, a cell or subject is contacted with an agent suspected or known to have BACE1 polypeptide expression modulating activity. The change in BACE1 polypeptide gene expression is then measured as compared to a control or standard sample. The control or standard sample can be the baseline expression of the cell or subject prior to contact with the agent. An agent that modulates BACE1 polypeptide gene expression can be a polynucleotide, for example, an antisense molecule, a triplex agent, a ribozyme, or a double stranded interfering RNA that interacts with a BACE1. For example, an antisense molecule can be directed to the structural gene region or to the promoter region of a BACE1 gene. The agent also can be a peptide, peptidomimetic, antibody, or small organic molecule, and can act as an agonist or antagonist of BACE1 activity.

Double stranded interfering RNA molecules are especially useful to inhibit expression of a target gene. For example, double stranded RNA molecules can be injected into a target cell or organism to inhibit expression of a gene and the resultant gene products activity. It has been found that such double stranded RNA molecules are more effective at inhibiting expression than either RNA strand alone. (Fire *et al.*, Nature, 1998, 19:391(6669):806-11).

When a disorder is associated with abnormal expression of a BACE1 polypeptide (*e.g.*, overexpression, or expression of a mutated form of the protein) or as a result of expression of a substrate for the BACE1 polypeptide, a therapeutic approach which directly interferes with the translation of a BACE1 polypeptide is possible.

Alternatively, similar methodology may be used to study gene activity. For example, antisense nucleic acid, double stranded interfering RNA or ribozymes could be used to bind to a BACE1 polypeptide mRNA sequence or to cleave it.

Antisense RNA or DNA molecules bind specifically with mRNA expressed from a targeted gene, interrupting the expression of the gene product (protein). The antisense binds to the messenger RNA forming a double stranded molecule which cannot be translated by the cell. Antisense oligonucleotides of about 15-25 nucleotides are preferred since they are easily synthesized and have an inhibitory effect just like antisense RNA molecules. In addition, chemically reactive groups, such as iron-linked ethylenediaminetetraacetic acid (EDTA-Fe) can be attached to an antisense oligonucleotide, causing cleavage of the RNA at the site of hybridization. Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, *Scientific American*, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target BACE1 polypeptide producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.*, 172:289, 1988).

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher et al., *Antisense Res. and Dev.*, 1:227, 1991; Helene, *Anticancer Drug Design*, 6:569, 1991).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes, tetrahymena-type (Hasselhoff, Nature, 334:585, 1988) and "hammerhead"-type. Tetrahymena-type ribozymes recognize sequences which are four bases in length, while hammerhead-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to tetrahymena-type ribozymes for inactivating a specific mRNA species and 18-base recognition sequences are preferable to shorter recognition sequences. These and other uses of antisense and ribozymes methods to inhibit the *in vivo* translation of genes are known in the art (*e.g.*, De Mesmaeker et al., Curr. Opin. Struct. Biol., 5:343, 1995; Gewirtz et al., Proc. Natl. Acad. Sci. U.S.A., 93:3161, 1996b; Stein, Chem. and Biol. 3:319, 1996).

Delivery of antisense, triplex agents, ribozymes, competitive inhibitors, double stranded interfering RNA and the like can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system or by injection. Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a polynucleotide sequence of interest into the viral

vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing, for example, an antisense polynucleotide.

Another targeted delivery system for polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0  $\mu\text{m}$  can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley et al., Trends Biochem. Sci., 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al., BioTechniques, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of

divalent cations. Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidyl-glycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticuloendothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. In general, the compounds bound to the surface of the targeted delivery system will be ligands and receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound, such as a receptor.

The agents useful in the method of the invention can be administered, for *in vivo* application, parenterally by injection or by gradual perfusion over time. Administration may be intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or

transdermally. For *in vitro* studies the agents may be added or dissolved in an appropriate biologically acceptable buffer and added to a cell or tissue.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents and inert gases and the like.

It is envisioned that the invention can be used to treat pathologies associated with neurodegenerative diseases and associated disorders, A $\beta$ 11-40/42 accumulation diseases such as Alzheimer's disease. Therefore, the present invention encompasses methods for ameliorating a disorder associated with neurodegenerative disorders, including treating a subject having the disorder, at the site of the disorder, with an agent which modulates a BACE1 expression or activity or its interaction with its substrate (*e.g.*, APP). Generally, the terms "treating", "treatment" and the like are used herein to mean affecting a subject, tissue or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for an infection or disease and/or adverse effect attributable to the infection or disease.

"Treating" as used herein encompasses any treatment of, or prevention of a disease in an invertebrate, a vertebrate, a mammal, particularly a human, and includes: (a) preventing the disorder from occurring in a subject that may be predisposed to the disorder, but has not yet been diagnosed as having it; (b) inhibiting the disorder, *i.e.*, arresting its development; or (c) relieving or ameliorating the disorder, *i.e.*, cause regression of the disorder. By "A $\beta$ 11-40/42 accumulation disease" is meant a disease that is characterized as having an increase in A $\beta$ 11-40 and A $\beta$ 11-42 peptides over normal

levels. Such accumulations in APP fragments lead to degenerative diseases that include, for example, Alzheimer's disease.

The present invention provides various compositions that can be administered to an individual and are useful for ameliorating symptoms attributable to a BACE1 or APP processing associated disorder. A composition according to one embodiment of the invention is prepared by formulating an anti-BACE1 antibody, a polypeptide or peptide derivative of a BACE1 polypeptide, a BACE1 polypeptide mimetic, a drug, chemical or combination of chemicals, a BACE1 polypeptide-modulating agent, or a combination thereof into a form suitable for administration to a subject using carriers, excipients and additives or auxiliaries. Frequently used carriers or auxiliaries include magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talc, milk protein, gelatin, starch, vitamins, cellulose and its derivatives, animal and vegetable oils, polyethylene glycols and solvents, such as sterile water, alcohols, glycerol and polyhydric alcohols. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. Other physiologically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like (see, for example, Remington's Pharmaceutical Sciences, 15th ed. Easton: Mack Publishing Co., 1405-1412, 1461-1487 (1975) and The National Formulary XIV., 14th ed. Washington: American Pharmaceutical Association (1975), each of which is incorporated herein by reference). The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art (see Goodman and Gilman, The Pharmacological Basis for Therapeutics 7th ed.).

A composition of the invention generally, but not necessarily, is prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a subject, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the subject, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by

single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The compositions according to the invention can be administered locally or systemically in a therapeutically effective dose. Amounts effective for this use will depend, in part, on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, for example, in Langer, Science, 249:1527, (1990); Gilman et al. (eds.) (1990). Administration of a composition of the invention can be accomplished by any means known to the skilled artisan, and preferably is administered to a vertebrate organism, particularly a mammal, including a human.

An anti-BACE1 antibody can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. Physiologically acceptable carrier preparations for parenteral administration include sterile or aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers for occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners and elixirs containing inert diluents commonly used in the art, such as purified water.

In another embodiment, the invention provides a method for identifying an agent which interacts with or modulates expression or activity of a BACE1 polypeptide including

incubating components comprising an agent and a BACE1 polypeptide, or a recombinant cell expressing a BACE1 polypeptide, under conditions sufficient to allow the agent to interact and determining the effect of the agent on the expression or activity of the gene or polypeptide, respectively. The effect can be any means by which gene expression or protein activity is modulated, and includes measuring the interaction of the agent with the BACE1 protein by physical means including, for example, fluorescence detection of the binding of the protein to a substrate or binding agent. Such agents can include, for example, polypeptides, peptidomimetics, chemical compounds, small molecules and biologic agents as described below.

Incubating includes conditions which allow contact between the test agent and a BACE1 polypeptide, a cell expressing a BACE1 polypeptide or nucleic acid encoding a BACE1 polypeptide. Contacting includes in solution and in solid phase. The test agent may optionally be a combinatorial library for screening a plurality of agents. Agents identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki et al., *BioTechnology*, 3:1008-1012, 1985), oligonucleotide ligation assays (OLAs; Landegren et al., *Science*, 241:1077, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren et al., *Science*, 242:229-237, 1988). Thus, the methods of the invention includes combinatorial chemistry methods for identifying chemical agents that bind to or affect BACE1 polypeptide expression or activity.

Areas of investigation are the development of therapeutic treatments. The screening identifies agents that provide modulation of BACE1 polypeptide function in targeted organisms. Of particular interest are screening assays for agents that have a low toxicity or a reduced number of side effects for humans. In particular, since the invention provides for the first time that BACE1 activity is species specific and results in the formation of an A $\beta$ 11-40/42 product, detection of the effect of an agent on product formation can be easily assayed and thus the identification of potential therapeutics is provided by the present invention.

The term "agent" as used herein refers to any molecule that can alter or mimic the physiological function or expression of a BACE1 polypeptide. Thus, an agent can be a peptide or polypeptide, a polynucleotide, a polysaccharide, a peptidomimetic, a small organic molecule, or a combination thereof, for example, a nucleoprotein or lipoprotein. Generally, a plurality of assay mixtures are run in parallel with different agents or different concentrations of an agent to obtain a differential response to the various concentrations. Typically, a negative control, *i.e.*, no agent or an amount that produces a result below the level of detection is included and, where available, one or more positive controls is included.

In a further embodiment, the invention provides a method of detecting a BACE1 or APP fragments (*e.g.*, A $\beta$ 11-40/42), a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42) polypeptide or a BACE1 polynucleotide or diagnosing a BACE1 or APP fragments (*e.g.*, A $\beta$ 11-40/42)-related disorder (*e.g.*, AD) in a subject including contacting a sample (*e.g.*, blood, serum, cerebrospinal fluid or a cellular sample, or tissue sample) suspected of containing a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42) polypeptide or a BACE1 polynucleotide with a reagent which binds to the polypeptide or polynucleotide (herein after sample). The sample can be or contain a nucleic acid, such as DNA or RNA, or a protein. When the sample contains a nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the sample contains protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Additional labels suitable for binding to an antibody or nucleic acid probe are known in the art or can be ascertained using routine experimentation. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds. In addition, the antibodies, polypeptides and polynucleotide sequences of the invention can be used to diagnosis a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42)-related disorder.

An antiserum, polyclonal antibody or monoclonal antibody of the invention, which specifically binds a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42) polypeptide is useful for the *in vivo* and *in vitro* detection of antigen. The detectably labeled monoclonal antibody is given in a dose, which is diagnostically effective. A diagnostically effective amount is an amount of a detectably labeled monoclonal antibody that is sufficient to enable detection of a BACE1 or APP fragments (*e.g.*, A $\beta$ 11-40/42) or a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42) polypeptide antigen for which the monoclonal antibodies are specific.

The concentration of a detectably labeled monoclonal antibody administered to a subject should be sufficient such that the binding to those cells, body fluid, or tissue having a BACE1 or APP (*e.g.*, A $\beta$ 11-40/42) polypeptide that is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay, which is detectable for a given type of instrument. Another factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope is long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which can be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are  $^{111}\text{In}$ ,  $^{97}\text{Ru}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{72}\text{As}$ ,  $^{89}\text{Zr}$ , and  $^{201}\text{Tl}$ .

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements, which are particularly useful in such techniques, include  $^{157}\text{Gd}$ ,  $^{55}\text{Mn}$ ,  $^{162}\text{Dy}$ ,  $^{52}\text{Cr}$ , and  $^{56}\text{Fe}$ .

In another embodiment, nucleic acid probes can be used to identify a BACE1 polynucleotide from a sample obtained from a subject. Examples of specimens from which nucleic acid sequence encoding a BACE1 polypeptide can be derived include insect, human, primate, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine, opine and bovine species. Such probes also can be used to identify a polynucleotide encoding aBACE1 polypeptide from a specimen obtained from a subject. Examples of specimens from which nucleic acid sequence encoding a BACE1 polypeptide can be derived include human, primate, swine, porcine, feline, canine, equine, murine, cervine, caprine, lupine, leporidine and bovine species.

Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double stranded DNA. For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. By using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace et al., Nucl. Acid Res. 9:879, 1981).

In an embodiment of the invention, purified nucleic acid fragments containing intervening sequences or oligonucleotide sequences of 10-50 base pairs are radioactively labeled. The labeled preparations are used to probe nucleic acids from a specimen by the Southern hybridization technique. Nucleotide fragments from a specimen, before or after amplification, are separated into fragments of different molecular masses by gel electrophoresis and transferred to filters that bind nucleic acid. After exposure to the labeled probe, which will hybridize to nucleotide fragments containing target nucleic acid sequences, binding of the radioactive probe to target nucleic acid fragments is identified by autoradiography (see Genetic Engineering, 1, ed. Robert Williamson, Academic Press, (1981), 72-81). Alternatively, nucleic acid from the specimen can be bound directly to filters to which the radioactive probe selectively attaches by binding nucleic acids having the sequence of interest. Specific sequences and the degree of binding is quantitated by directly counting the radioactive emissions.

Where the target nucleic acid is not amplified, detection using an appropriate hybridization probe may be performed directly on the separated nucleic acid. In those instances where the target nucleic acid is amplified, detection with the appropriate hybridization probe would be performed after amplification.

For the most part, the probe will be detectably labeled with an atom or inorganic radical, most commonly using radionuclides, but also heavy metals can be used. Conveniently, a radioactive label may be employed. Radioactive labels include  $^{32}\text{P}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{111}\text{In}$ ,  $^{99}\text{Tc}$ , or the like. Any radioactive label may be employed which provides for an adequate signal and has sufficient half-life. Other labels include ligands, which can serve as a specific binding pair member for a labeled ligand, and the like. A wide variety of labels routinely employed in immunoassays can readily be employed in the present assay. The choice of the label will be governed by the effect of the label on the rate of hybridization and binding of the probe to a nucleotide sequence. It will be necessary that the label provide sufficient sensitivity to detect the amount of a nucleotide sequence available for hybridization.

The manner in which the label is bound to the probe will vary depending upon the nature of the label. For a radioactive label, a wide variety of techniques can be employed. Commonly employed is nick translation with an a  $^{32}\text{P}$ -dNTP or terminal phosphate hydrolysis with alkaline phosphatase followed by labeling with radioactive  $^{32}\text{P}$  employing  $^{32}\text{P}$ -NTP and T4 polynucleotide kinase. Alternatively, nucleotides can be synthesized where one or more of the elements present are replaced with a radioactive isotope, for example, the replacement of hydrogen-1 with tritium (H-3). If desired, complementary labeled strands can be used as probes to enhance the concentration of hybridized label.

Standard hybridization techniques for detecting a nucleic acid sequence are known in the art. The particular hybridization technique is not essential to the invention. Other hybridization techniques are described by Gall and Pardue, *Proc. Natl. Acad. Sci.* 63:378, 1969); and John et al., *Nature*, 223:582, 1969). As improvements are made in hybridization techniques they can readily be applied in the method of the invention.

The amount of labeled probe present in the hybridization solution will vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the filter, and the stringency of the hybridization. Generally, substantial excess over stoichiometric concentrations of the probe will be employed to enhance the rate of binding of the probe to the fixed target nucleic acid.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means containing one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. One of the container means may comprise a probe which is or can be detectably labeled. Such probe may be a nucleic acid sequence specific for BACE1; or antibodies specific for BACE1, fragments thereof; or APP or fragments thereof. The kit also can include a container comprising a reporter-means, such as an enzymatic, fluorescent, or radionuclide label to identify the detectably labeled oligonucleotide probe or antibody. Where the kit utilizes nucleic acid hybridization to detect the target nucleic

acid, the kit may also have containers containing nucleotide(s) for amplification of the target nucleic acid sequence.

Various methods to make the transgenic non-human animals of the invention can be employed. Generally speaking, three such methods may be employed. In one such method, an embryo at the pronuclear stage (a "one cell embryo") is harvested from a female and the transgene is microinjected into the embryo, in which case the transgene will be chromosomally integrated into both the germ cells and somatic cells of the resulting mature animal. In another such method, embryonic stem cells are isolated and the transgene incorporated therein by electroporation, plasmid transfection or microinjection, followed by reintroduction of the stem cells into the embryo where they colonize and contribute to the germ line. Methods for microinjection of mammalian species is described in U.S. Pat. No. 4,873,191.

In another method, embryonic cells are infected with a retrovirus containing the transgene whereby the germ cells of the embryo have the transgene chromosomally integrated therein. When the animals to be made transgenic are avian, because avian fertilized ova generally go through cell division for the first twenty hours in the oviduct, microinjection into the pronucleus of the fertilized egg is problematic due to the inaccessibility of the pronucleus. Therefore, of the methods to make transgenic animals described generally above, retrovirus infection is preferred for avian species, for example as described in U.S. Pat No. 5,162,215. If microinjection is to be used with avian species, however, a published procedure by Love et al., (Biotechnology, 12, Jan 1994) can be utilized whereby the embryo is obtained from a sacrificed hen approximately two and one-half hours after the laying of the previously laid egg, the transgene is microinjected into the cytoplasm of the germinal disc and the embryo is cultured in a host shell until maturity. When the animals to be made transgenic are bovine or porcine, microinjection can be hampered by the opacity of the ova thereby making the nuclei difficult to identify by traditional differential interference-contrast microscopy. To overcome this problem, the ova can first be centrifuged to segregate the pronuclei for better visualization.

The non-human transgenic animals of the invention can be any vertebrate, including, for example, bovine, porcine, ovine and avian animals. The transgenic non-human animals of the invention are produced by introducing at least one transgene into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for microinjection. The use of zygotes as is target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

The term "transgenic" is used to describe an animal which includes exogenous genetic material within all of its cells. A transgenic animal can be produced by cross-breeding two chimeric animals which include exogenous genetic material within cells used in reproduction. Twenty-five percent of the resulting offspring will be transgenic *i.e.*, animals which include the exogenous genetic material within all of their cells in both alleles, 50% of the resulting animals will include the exogenous genetic material within one allele and 25% will include no exogenous genetic material.

In the microinjection method useful in the practice of the subject invention, the transgene is digested and purified free from any vector DNA, *e.g.*, by gel electrophoresis. It is preferred that the transgene include an operatively associated promoter which interacts with cellular proteins involved in transcription, ultimately resulting in constitutive expression. Promoters useful in this regard include those from cytomegalovirus (CMV), Moloney leukemia virus (MLV), and herpes virus, as well as those from the genes encoding metallothionin, skeletal actin, P-enolpyruvate carboxylase (PEPCK), phosphoglycerate (PGK), DHFR, and thymidine kinase. Promoters for viral long terminal repeats (LTRs) such as Rous Sarcoma Virus can

also be employed. When the animals to be made transgenic are avian, preferred promoters include those for the chicken  $\beta$ -globin gene, chicken lysozyme gene, and avian leukosis virus. Constructs useful in plasmid transfection of embryonic stem cells will employ additional regulatory elements well known in the art such as enhancer elements to stimulate transcription, splice acceptors, termination and polyadenylation signals, and ribosome binding sites to permit translation.

Retroviral infection can also be used to introduce transgene into a non-human animal, as described above. The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, Proc. Natl. Acad. Sci USA 73:1260-1264, 1976). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan et al. (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retro virus carrying the transgene (Jahner et al., Proc. Natl. Acad. Sci. USA 82: 6927-6931, 1985; Van der Putten et al., Proc. Natl. Acad. Sci USA 82: 6148-6152, 1985). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, et al., EMBO J. 6: 383-388, 1987). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al., Nature 298: 623-628, 1982). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic nonhuman animal. Further, the founder may contain various retro viral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner et al., *supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al., Nature 292:154-156, 1981; Bradley et al., Nature 309:255-258, 1984; Gossler et al., Proc. Natl. Acad. Sci USA 83:9065-9069, 1986; and Robertson

et al., Nature 322:445-448, 1986). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retro virus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a nonhuman animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal (see Jaenisch, Science 240:1468-1474, 1988).

The transgene can be any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism, i.e., is either stably integrated or as a stable extrachromosomal element, that develops from that cell. Such a transgene can be a gene that is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or can represent a gene homologous to an endogenous gene of the organism. Included within this definition is a transgene created by the providing of an RNA sequence that is transcribed into DNA and then incorporated into the genome. The transgenes of the invention include DNA sequences which encode BACE1 or a selectable marker flanked by regions of sequence having homology to BACE1, and include polynucleotides, which may be expressed in a transgenic non-human animal.

The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" or "knockout" is used herein to refer to the targeted disruption of a gene *in vivo* with substantially complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. Any transgenic technology as disclosed herein or otherwise known in the art can be used to produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional (knocked out).

After an embryo has been microinjected, colonized with transfected embryonic stem cells or infected with a retrovirus containing the transgene (except for practice of the

subject invention in avian species which is addressed elsewhere herein) the embryo is implanted into the oviduct of a pseudopregnant female. The consequent progeny are tested for incorporation of the transgene by Southern blot analysis of blood or tissue samples using transgene specific probes. PCR is particularly useful in this regard. Positive progeny (G0) are crossbred to produce offspring (G1) which are analyzed for transgene expression by northern blot analysis of tissue samples.

As used herein, a "heterologous gene" or "heterologous polynucleotide sequence" is defined in relation to the transgenic non-human organism producing or containing such a gene product. A heterologous polypeptide is defined as a polypeptide having an amino acid sequence or an encoding DNA sequence corresponding to that of a heterologous gene not normally found in an organism. The term "heterologous" also is used in reference to two or more polynucleotides or polypeptides, wherein one of the molecules can be considered a reference molecule, and the second or other is compared thereto. As such, a first polynucleotide is considered heterologous to a second polynucleotide, for example, if the two polynucleotides are not normally associated with each other in a cell of an organism.

As used herein, the term "targeting construct" refers to a polynucleotide which comprises: (1) at least one homology region having a sequence that is substantially identical to or substantially complementary to a sequence present in a host cell endogenous gene locus, and (2) a targeting region which becomes integrated into an host cell endogenous gene locus by homologous recombination between a targeting construct homology region and the endogenous gene locus sequence. A transiently incorporated targeting construct is one that is incorporated into the endogenous gene locus and is eliminated from the host genome by selection. A targeting region may comprise a sequence that is substantially homologous to an endogenous gene sequence and/or may comprise a non-homologous sequence, such as a selectable marker (*e.g.*, *neo*, *tk*, *gpt*). The term "targeting construct" does not necessarily indicate that the polynucleotide comprises a gene which becomes integrated into the host genome, nor does it necessarily indicate that the polynucleotide comprises a

complete structural gene sequence. As used in the art, the term "targeting construct" is synonymous with the term "targeting transgene" as used herein.

The term "homology region" as used herein refers to a segment (*i.e.*, a portion) of a targeting construct having a sequence that substantially corresponds to, or is substantially complementary to, a predetermined endogenous gene sequence, which can include sequences flanking said gene. A homology region is generally at least about 100 nucleotides long, preferably at least about 250 to 500 nucleotides long, typically at least about 1000 nucleotides long or longer. Although there is no demonstrated theoretical minimum length for a homology region to mediate homologous recombination, it is believed that homologous recombination efficiency generally increases with the length of the homology region. Similarly, the recombination efficiency increases with the degree of sequence homology between a targeting construct homology region and the endogenous target sequence, with optimal recombination efficiency occurring when a homology region is isogenic with the endogenous target sequence. A homology region does not necessarily denote formation of a base-paired hybrid structure with an endogenous sequence. Endogenous gene sequences that substantially correspond to, or are substantially complementary to, a transgene homology region are referred to herein as "crossover target sequences" or "endogenous target sequences."

As used herein, the term "transcriptional unit" or "transcriptional complex" refers to a polynucleotide sequence that comprises a structural gene (exons), a cis-acting linked regulatory element (*e.g.*, a promoter or enhancer sequence) and other cis-acting sequences necessary for efficient transcription of the structural sequences, distal regulatory elements necessary for appropriate tissue-specific and developmental transcription of the structural sequences, and additional cis sequences important for efficient transcription and translation (*e.g.*, polyadenylation site, mRNA stability controlling sequences).

A correctly targeted construct integrates within or adjacent to an endogenous crossover target sequence, such as a portion of an endogenous BACE1 gene locus.

For example, a targeting transgene encoding neo and which is flanked by homology regions having substantial identity with endogenous BACE1 gene sequences of the first exon of BACE1 is correctly targeted when the transgene portion is integrated into a chromosomal location so as to replace, for example, the first exon of the endogenous BACE1 gene. It is possible to generate cells having both a correctly targeted transgene(s) and an incorrectly targeted transgene(s). Cells and animals having a correctly targeted transgene(s) and/or an incorrectly targeted transgene(s) may be identified and resolved by PCR and/or Southern blot analysis of genomic DNA.

As used herein, the term "targeting region" refers to a portion of a targeting construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a homology region and an endogenous BACE1 gene sequence. Typically, a targeting region is flanked on each side by a homology region, such that a double crossover recombination between each of the homology regions and their corresponding endogenous BACE1 gene sequences results in replacement of the portion of the endogenous BACE1 gene locus by the targeting region; in such double crossover gene replacement targeting constructs the targeting region can be referred to as a "replacement region". However, some targeting constructs may employ only a single homology region.

As used herein, the term "replacement region" refers to a portion of a targeting construct flanked by homology regions. Upon double crossover homologous recombination between flanking homology regions and their corresponding endogenous BACE1 gene crossover target sequences, the replacement region is integrated into the host cell chromosome between the endogenous crossover target sequences. Replacement regions can be homologous (*e.g.*, have a sequence similar to the endogenous BACE1 gene sequence but having a point mutation or missense mutation), non-homologous (*e.g.*, a *neo* gene expression cassette), or a combination of homologous and non-homologous regions. The replacement region can convert the endogenous BACE1 allele into a mutant BACE1 allele comprising a point mutation

or missense mutation or disrupt the BACE1 allele by integrating a non-homologous transgene at the BACE1 allele.

The terms "functional disruption" or "functionally disrupted" as used herein means that a gene locus comprises at least one mutation or structural alteration such that the functionally disrupted gene is incapable of directing the efficient expression of functional gene product. For example, an endogenous BACE1 gene that has a *neo* gene cassette integrated into an exon of a BACE1 gene, is not capable of encoding a functional protein and is therefore a functionally disrupted BACE1 gene locus. In addition, a targeted mutation in an exon of an endogenous BACE1 gene may result in a mutated endogenous gene that can express a truncated BACE1 protein that is non-functional. Functional disruption can include the complete substitution of a heterologous BACE1 gene locus in place of an endogenous BACE1 locus, so that, for example, a targeting transgene that replaces the entire mouse BACE1 locus with a human BACE1 allele, which may be functional in the mouse, is said to have functionally disrupted the endogenous murine BACE1 locus by displacing it. Preferably, at least one exon which is incorporated into the mRNAs encoding most or all of the BACE1 isoforms are functionally disrupted. Deletion or interruption of essential transcriptional regulatory elements, polyadenylation signal(s), splicing site sequences will also yield a functionally disrupted gene.

Functional disruption of an endogenous BACE1 gene also can be produced by other methods (*e.g.*, antisense polynucleotide gene suppression). The term "structurally disrupted" refers to a targeted gene wherein at least one structural sequence (*e.g.*, an exon sequence) has been altered by homologous gene targeting (*e.g.*, by insertion, deletion, point mutation(s), and/or rearrangement). Typically, BACE1 alleles that are structurally disrupted are consequently functionally disrupted, however BACE1 alleles may also be functionally disrupted without concomitantly being structurally disrupted, *i.e.*, by targeted alteration of a non-exon sequence such as ablation of a promoter. An allele comprising a targeted alteration that interferes with the efficient expression of a functional gene product from the allele is referred to in the art as a "null allele" or "knockout allele".

As used herein, "isoform", "BACE1", and "BACE1 isoform" refer to a polypeptide that is encoded by at least one exon and includes a sequence as set forth in GenBank Accession No. AF190725 (Vassar et al., Science 286:735, 1999; see, also GenBank Accession No. AAF04142, for BACE1 amino acid sequence). A BACE isoform can be encoded by any BACE allele (or exon thereof) that is associated with a form of Alzheimer's disease or that is not associated with an AD disease phenotype. In some embodiments, the endogenous non-human BACE1 alleles are functionally disrupted so that expression of endogenously encoded BACE1 is suppressed or eliminated. In one variation, an endogenous BACE1 allele is targeted for disruption by homologous recombination.

Gene targeting, which is a method of using homologous recombination to modify a mammalian genome, can be used to introduce changes into cultured cells. By targeting a gene of interest in embryonic stem (ES) cells, these changes can be introduced into the germ lines of laboratory animals to study the effects of the modifications on whole organisms, among other uses. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that has a segment homologous to a target locus and which also comprises an intended sequence modification (*e.g.*, insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted. A common scheme to disrupt gene function by gene targeting in ES cells is to construct a targeting construct which is designed to undergo a homologous recombination with its chromosomal counterpart in the ES cell genome. The targeting constructs are typically arranged so that they insert additional sequences, such as a selectable marker, into coding elements of the target gene, thereby functionally disrupting it. Targeting constructs usually are insertion-type or replacement-type constructs (Hasty et al., Mol. Cell. Biol. 11:4509, 1991).

The invention encompasses methods to produce non-human animals (*e.g.*, non-primate mammals) that have the endogenous BACE1 gene inactivated by gene targeting with a homologous recombination targeting construct. Typically, a non-

human BACE1 gene sequence is used as a basis for producing PCR primers that flank a region that will be used as a homology region in a targeting construct. The PCR primers are then used to amplify, by high fidelity PCR amplification (Mattila et al., Nucleic Acids Res. 19:4967, 1991; Eckert and Kunkel, PCR Methods and Applications 1:17, 1991; U.S. Pat. No. 4,683,202, which are incorporated herein by reference), a genomic sequence from a genomic clone library or from a preparation of genomic DNA, preferably from the strain of non-human animal that is to be targeted with the targeting construct. The amplified DNA is then used as a homology region and/or targeting region. Thus, homology regions for targeting a non-human BACE1 gene may be readily produced on the basis of nucleotide sequence information available in the art and/or by routine cloning (*e.g.*, GenBank Acc. No. AF190725). General principles regarding the construction of targeting constructs and selection methods are reviewed in Bradley et al., Bio/Technology 10:534, 1992, incorporated herein by reference). In addition, to the disruption of endogenous non-human BACE1 genes the transgenic organism may include one or more transgenes encoding for example APP comprising the Swedish mutation.

Targeting constructs can be transferred into pluripotent stem cells, such as murine embryonal stem cells, wherein the targeting constructs homologously recombine with a portion of an endogenous BACE1 gene locus and create mutation(s) (*i.e.*, insertions, deletions, rearrangements, sequence replacements, and/or point mutations) which prevent the functional expression of the endogenous BACE1 gene.

A preferred method of the invention is to delete, by targeted homologous recombination, essential structural elements of the endogenous BACE1 gene. For example, a targeting construct can homologously recombine with an endogenous BACE1 gene and delete a portion spanning substantially all of one or more of the exons to create an exon-depleted allele, typically by inserting a replacement region lacking the corresponding exon(s). Transgenic animals homozygous for the exon-depleted allele (*e.g.*, by breeding of heterozygotes to each other) produce cells which are essentially incapable of expressing a functional endogenous BACE1 polypeptide (preferably incapable of expressing any of the naturally-occurring isoforms).

Similarly, homologous gene targeting can be used, if desired, to functionally disrupt a BACE1 gene by deleting only a portion of an exon.

Targeting constructs can also be used to delete essential regulatory elements of an endogenous BACE1 gene, such as promoters, enhancers, splice sites, polyadenylation sites, and other regulatory sequences, including cis-acting sequences that occur upstream or downstream of the BACE1 structural gene but which participate in endogenous BACE1 gene expression. Deletion of regulatory elements is typically accomplished by inserting, by homologous double crossover recombination, a replacement region lacking the corresponding regulatory element(s).

Another method of the invention is to interrupt essential structural and/or regulatory elements of an endogenous BACE1 gene by targeted insertion of a polynucleotide sequence, and thereby functionally disrupt the endogenous BCE1 gene. For example, a targeting construct can homologously recombine with an endogenous BACE1 gene and insert a non-homologous sequence, such as a *neo* expression cassette, into a structural element (*e.g.*, an exon) and/or regulatory element (*e.g.*, enhancer, promoter, splice site, polyadenylation site) to yield a targeted BCE1 allele having an insertional interruption. The inserted sequence can range in size from about 1 nucleotide (*e.g.*, to produce a frame shift in an exon sequence) to several kilobases or more, as limited by efficiency of homologous gene targeting with targeting constructs having a long non-homologous replacement region. Targeting constructs of the invention can also be employed to replace a portion of an endogenous BACE1 gene with an exogenous sequence (*i.e.*, a portion of a targeting transgene); for example, an exon of a BACE1 gene may be replaced with a substantially identical portion that contains a nonsense or missense mutation.

In one embodiment, inactivation of an endogenous murine BACE1 locus is achieved by targeted disruption of the appropriate gene by homologous recombination in a mouse embryonic stem cell. For inactivation, any targeting construct that produces a genetic alteration in the target BACE1 gene locus resulting in the prevention of effective expression of a functional gene product of that locus may be employed. If

only regulatory elements are targeted, some low-level expression of the targeted gene may occur (*i.e.*, the targeted allele is "leaky"), however the level of expression may be sufficiently low that the leaky targeted allele is functionally disrupted.

In another embodiment of the invention, an endogenous BACE1 gene in a non-human host is functionally disrupted by homologous recombination with a targeting construct that does not comprise a functionally equivalent sequence. In this embodiment, a portion of the targeting construct integrates into an essential structural or regulatory element of the endogenous BACE1 gene locus, thereby functionally disrupting it to generate a null allele. Typically, null alleles are produced by integrating a non-homologous sequence encoding a selectable marker (*e.g.*, a *neo* gene expression cassette) into an essential structural and/or regulatory sequence of a BACE1 gene by homologous recombination of the targeting construct homology regions with endogenous BACE1 gene sequences, although other strategies may be employed.

Generally, a targeting construct is transferred by electroporation or microinjection into a totipotent embryonal stem (ES) cell line, such as the murine AB-1 or CCE lines. The targeting construct homologously recombines with endogenous sequences in or flanking an BACE1 gene locus and functionally disrupts at least one allele of the BACE1 gene. Typically, homologous recombination of the targeting construct with endogenous BACE1 locus sequences results in integration of a non-homologous sequence encoding a selectable marker, such as *neo*, usually in the form of a positive selection cassette. The functionally disrupted allele is termed an BACE1 null allele. ES cells having at least one BACE1 null allele are selected for by propagating the cells in a medium that permits the preferential propagation of cells expressing the selectable marker. Selected ES cells are examined by PCR analysis and/or Southern blot analysis to verify the presence of a correctly targeted BACE1 allele.

Breeding of non-human animals which are heterozygous for a null allele may be performed to produce non-human animals homozygous for said null allele, *i.e.*, "knockout" animals (Donehower et al., *Nature* 256:215, 1992, which is incorporated herein by reference). In some instances, breeding animals to maintain heterozygosity

may be desired. As described more fully below, the transgenic organisms of the invention have utility as both heterozygous and homozygous BACE1 null alleles. Alternatively, ES cells homozygous for a null allele having an integrated selectable marker can be produced in culture by selection in a medium containing high levels of the selection agent (*e.g.*, G418 or hygromycin). Heterozygosity and/or homozygosity for a correctly targeted null allele can be verified with PCR analysis and/or Southern blot analysis of DNA isolated from an aliquot of a selected ES cell clone and/or from tail biopsies.

If desired, a transgene encoding, for example, a heterologous APP polypeptide comprising the Swedish mutation can be transferred into a non-human host having a BACE1 null allele, preferably into a non-human ES cell that is homozygous for the BACE1 null allele. It is generally advantageous that the transgene comprises a promoter and enhancer which drive expression of structural sequences encoding a functional heterologous Swedish mutation APP gene product. Thus, for example, a knockout mouse homozygous for null alleles at the BACE1 locus can serve as a host for a transgene which encodes and expresses a gene associated with an Alzheimer's disease associated phenotype.

Several gene targeting techniques have been described, including but not limited to co-electroporation, single crossover integration, and double crossover recombination (Bradley et al., *BioTechnology* 10:534, 1992). The invention can be practiced using essentially any applicable homologous gene targeting strategy known in the art. The configuration of a targeting construct depends upon the specific targeting technique chosen. For example, a targeting construct for single crossover integration targeting need only have a single homology region linked to the targeting region, whereas a double crossover replacement-type targeting construct requires two homology regions, one flanking each side of the replacement region.

For example, in one embodiment a targeting construct comprising, in order: (1) a first homology region having a sequence substantially identical to a sequence within about 3 kilobases upstream (*i.e.*, in the direction opposite to the translational reading frame

of the exons) of an exon of an endogenous BACE1 gene, (2) a replacement region comprising a positive selectable marker (*e.g.*, a pgk promoter driving transcription of a neo gene), (3) a second homology region having a sequence substantially identical to a sequence within about 2 kilobases downstream of said exon of said endogenous BACE1 gene, and (4) a negative selectable marker (*e.g.*, a HSV tk promoter driving transcription of an HSV tk gene). Such a targeting construct is suitable for double crossover replacement recombination which deletes a portion of the endogenous BACE1 locus spanning the desired exon and replaces it with the replacement region having the positive selectable marker. If the deleted exon is essential for expression of a functional BACE1 gene product, the resultant exon-depleted allele is functionally disrupted and is termed a null allele.

Targeting constructs of the invention comprise at least one BACE1 homology region operatively linked to a targeting region. A homology region has a sequence which substantially corresponds to, or is substantially complementary to, an endogenous BACE1 gene sequence of a non-human host animal, and may comprise sequences flanking the BACE1 gene. Although no lower or upper size boundaries for recombinant homology regions for gene targeting have been identified in the art, the typical homology region is believed to be in the range between about 50 base pairs and several tens of kilobases. Thus, targeting constructs are generally at least about 50 to 100 nucleotides long, preferably at least about 250 to 500 nucleotides long, more preferably at least about 1000 to 2000 nucleotides long, or longer. Construct homology regions are generally at least about 50 to 100 bases long, preferably at least about 100 to 500 bases long, and more preferably at least about 750 to 2000 bases long. Homology regions of about 7 to 8 kilobases in length are preferred, with one preferred embodiment having a first homology region of about 7 kilobases flanking one side of a replacement region and a second homology region of about 1 kilobase flanking the other side of said replacement region.

The length of homology (*e.g.*, substantial identity) for a homology region may be selected at the discretion of the practitioner on the basis of the sequence composition and complexity of the endogenous BACE1 gene target sequence(s) and guidance

provided in the art. Targeting constructs have at least one homology region having a sequence that substantially corresponds to, or is substantially complementary to, an endogenous BACE1 gene sequence (*e.g.*, an exon sequence, an enhancer, a promoter, an intronic sequence, or a flanking sequence within about 3-20 kb of a BACE1 gene or BACE1 gene homologue). Such a targeting transgene homology region serves as a template for homologous pairing and recombination with substantially identical endogenous BACE1 gene sequence(s). In targeting constructs, such homology regions typically flank the replacement region, which is a region of the targeting construct that is to undergo replacement with the targeted endogenous BACE1 gene sequence. Thus, a segment of the targeting construct flanked by homology regions can replace a segment of an endogenous BACE1 gene sequence by double crossover homologous recombination. Homology regions and targeting regions are linked together in conventional linear polynucleotide linkage (5' to 3' phosphodiester backbone). Targeting constructs are generally double stranded DNA molecules, most usually linear.

Without wishing to be bound by any particular theory of homologous recombination or gene conversion, it is believed that in such a double crossover replacement recombination, a first homologous recombination (*e.g.*, strand exchange, strand pairing, strand scission, strand ligation) between a first targeting construct homology region and a first endogenous BACE1 gene sequence is accompanied by a second homologous recombination between a second targeting construct homology region and a second endogenous BACE1 gene sequence, thereby resulting in the portion of the targeting construct that was located between the two homology regions replacing the portion of the endogenous BACE1 that was located between the first and second endogenous BACE1 sequences. For this reason, homology regions are generally used in the same orientation (*i.e.*, the upstream direction is the same for each homology region of a transgene to avoid rearrangements). Double crossover replacement recombination thus can be used to delete a portion of an endogenous BACE1 gene and concomitantly transfer a non-homologous portion (*e.g.*, a *neo* gene expression cassette) into the corresponding chromosomal location. Double crossover recombination can also be used to add a non-homologous portion into an endogenous

BACE1 gene without deleting endogenous chromosomal portions. However, double crossover recombination can also be employed simply to delete a portion of an endogenous BACE1 gene sequence without transferring a non-homologous portion into the endogenous BACE1 gene. Upstream and/or downstream from the nonhomologous portion may be a gene which provides for identification of whether a double crossover homologous recombination has occurred; such a gene is typically the HSV tk gene which may be used for negative selection.

The positive selectable marker encodes a selectable marker which affords a means for selecting cells which have integrated targeting transgene sequences. The negative selectable marker encodes a selectable marker which affords a means for selecting cells which do not have an integrated copy of the negative selection expression cassette. Thus, by a combination positive-negative selection protocol, it is possible to select cells that have undergone homologous replacement recombination and incorporated the portion of the transgene between the homology regions (*i.e.*, the replacement region) into a chromosomal location by selecting for the presence of the positive marker and for the absence of the negative marker.

Preferred selectable markers for inclusion in the targeting constructs of the invention encode and express a selectable drug resistance marker and/or a HSV thymidine kinase enzyme. Suitable drug resistance genes include, for example: *gpt* (xanthine-guanine phosphoribosyltransferase), which can be selected for with mycophenolic acid; *neo* (neomycin phosphotransferase), which can be selected for with G418 or hygromycin; and DHFR (dihydrofolate reductase), which can be selected for with methotrexate (Mulligan and Berg (1981) Proc. Natl. Acad. Sci. (U.S.A.) 78: 2072; Southern and Berg (1982) J. Mol. Appl. Genet. 1: 327; each of which is incorporated herein by reference).

Selection for correctly targeted recombinants will generally employ at least positive selection, wherein a non-homologous expression cassette encodes and expresses a functional protein (*e.g.*, *neo* or *gpt*) that confers a selectable phenotype to targeted cells harboring the endogenously integrated sequence, so that, by addition of a

selection agent (*e.g.*, G418 or mycophenolic acid) such targeted cells have a growth or survival advantage over cells which do not have an integrated sequence.

It is preferable that selection for correctly targeted homologous recombinants also employ negative selection, so that cells bearing only non-homologous integration of the transgene are selected against. Typically, such negative selection techniques employ an expression cassette encoding the herpes simplex virus thymidine kinase gene (HSV tk) positioned in the transgene so that it integrates only by non-homologous recombination. Such positioning generally is accomplished by linking the HSV tk expression cassette (or other negative selection marker) distal to the recombinant homology regions so that double crossover replacement recombination of the homology regions transfers the positive selection expression cassette to a chromosomal location but does not transfer the HSV tk gene (or other negative selection marker) to a chromosomal location. A nucleoside analog, gancyclovir, which is preferentially toxic to cells expressing HSV tk, can be used as the negative selection agent, as it selects for cells which do not have an integrated HSV tk expression marker. FIAU may also be used as a selective agent to select for cells lacking HSV tk.

Generally, targeting constructs of the invention include: (1) a positive selection marker flanked by two homology regions that are substantially identical to host cell endogenous BACE1 gene sequences, and (2) a distal negative selection marker. However, targeting constructs which include only a positive selection marker can also be used. Typically, a targeting construct will contain a positive selection marker, which includes a *neo* gene linked downstream (*i.e.*, towards the carboxy-terminus of the encoded polypeptide in translational reading frame orientation) of a promoter such as the HSV tk promoter or the pgk promoter.

It is preferred that targeting constructs of the invention have homology regions that are highly homologous to the predetermined target endogenous DNA sequence(s), preferably isogenic (*i.e.*, identical sequence). Isogenic or nearly isogenic sequences may be obtained by genomic cloning or high-fidelity PCR amplification of genomic

DNA from the strain of non-human animals which are the source of the ES cells used in the gene targeting procedure.

For making transgenic non-human animals (which include homologously targeted non-human animals), embryonal stem cells (ES cells) are preferred. The embryonic stem cells described herein can be obtained and manipulated according to published procedures (Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed., IRL Press, Washington, D.C. (1987); Zjilstra et al., *Nature* 342:435-438 (1989); and Schwartzberg et al., *Science* 246:799-803 (1989), each of which is incorporated herein by reference). Murine ES cells, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley (1990) *Cell* 62:1073) essentially as described by Robertson (*supra*, 1987, pages 71-112) can be used for homologous gene targeting. Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) *Nature* 326: 292-295), the D3 line (Doetschman et al. (1985) *J. Embryol. Exp. Morph.* 37: 27-45), and the CCE line (Robertson et al. (1986) *Nature* 323: 445-448).

The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotency of the ES cells (*i.e.*, their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal). The blastocysts containing the injected ES cells are allowed to develop in the uteri of pseudopregnant nonhuman females and are born as chimeric mice. The resultant transgenic mice are chimeric for cells having inactivated endogenous BACE1 loci and are back-crossed and screened for the presence of the correctly targeted transgene(s) by PCR or Southern blot analysis on tail biopsy DNA of offspring so as to identify transgenic mice heterozygous for the inactivated BACE1 locus. By performing the appropriate crosses, it is possible to produce a transgenic non-human animal homozygous for functionally disrupted BACE1 alleles. Such transgenic animals are substantially incapable of making an endogenous BACE gene product.

Non-human animals comprising transgenes which are heterozygous null or homozygous null for BACE1 can be used commercially as controls or standards in the development of AD therapeutics and diagnostics. For example, it is contemplated that the BACE-knockout organisms of the invention can be used as controls in screens for agents having the effect of lowering A $\beta$  production and/or accumulation. Such agents can be developed as pharmaceuticals for treating abnormal APP processing and/or Alzheimer's disease, amongst other neurodegenerative conditions. Other uses include using cells (particularly neuronal cells) derived from the BACE1-knockout organisms for creating protein expression profiles between BACE1-knockout organisms and organisms of identical species having a phenotype associated with Alzheimer's disease.

The effect of test agents on test animals, including transgenic animals, may be measured in various specimens from the test animals. In all cases, it will be necessary to obtain a control value which is characteristic of the level of production of APP and A $\beta$  polypeptide and peptides in animals lacking a phenotype associated with AD. Accordingly, the transgenic animals of the invention (*e.g.*, BACE1 knockout organisms) provide an ideal source of control organisms for studying AD as well as for screening the effects of agents on organisms having an AD-associated phenotype. Once such control level is determined, test compounds can be administered to additional test animals, where deviation from the average control value indicates that the test compound had an effect on the  $\beta$ -secretase activity in the animal. Test substances which are considered positive, *i.e.*, likely to be beneficial in the treatment of Alzheimer's disease or other  $\beta$ -amyloid-related conditions, will be those which are able to reduce the level of ATF- $\beta$ APP production, preferably by at least 20%, more preferably by at least 50%, and most preferably by at least 80% or which display a phenotype substantially identical or superior to the phenotype of the BACE1-knockout organisms of the invention.

As used herein, the term "Alzheimer's disease-associated phenotype" includes the appearance in an organism of a progressive formation of insoluble amyloid plaques and vascular deposits of the 4 kDa amyloid  $\beta$ -peptide. In addition, the phenotype can

result in organisms displaying impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

The test agents can be any molecule, compound, or other substance which can be added to the cell culture or administered to the test animal without substantially interfering with cell or animal viability. Suitable test agents may be small molecules, biological polymers, such as polypeptides, polysaccharides, polynucleotides, and the like. The test compounds will typically be administered to transgenic animals at a dosage of from 1 ng/kg to 10 mg/kg, usually from 10 µg/kg to 1 mg/kg.

Test compounds which are able to inhibit secretion or animal production or generate a phenotype substantially identical to the BACE1-knockout organisms of the invention (*e.g.*, having a reduce or negligible amount Aβ1-40, Aβ1-42, Aβ11-40, Aβ11-42 peptides) are considered as candidates for further determinations of the ability to block β-amyloid production in animals and humans. Inhibition of secretion or production indicates that cleavage of βAPP at the amino-terminus of βAP has likely been at least partly blocked, reducing the amount of a processing intermediate available for conversion to β-amyloid peptide.

The present invention further comprises compositions incorporating a compound selected by the above-described method and including a physiologically acceptable carrier. Such compositions, which can be administered to an individual, contain a therapeutic or prophylactic amount of at least one compound identified by the method of the present invention. The carrier can be any compatible, non-toxic substance suitable to deliver the compounds, and the compositions can be administered as discussed above.

Transgenic organisms and/or effects of agents on organisms (*e.g.*, organisms having a phenotype associated with AD) can be screened for presence of the transgene or changes in AD phenotypes in several ways. For example, brain APP protein and RNA expression can be detected and analyzed and the copy number and/or level of expression are determined using methods known to those of skill in the art. The

transgenic animals or organisms displaying a phenotype associated with AD can also be observed for clinical changes. Examples of neurobehavioral disorders for evaluation are poor mating response, agitation, diminished exploratory behavior in a novel setting, inactivity, seizures and premature death.

For a particular strain, organism or transgene, sufficient copies of an APP gene and/or a sufficient level of expression of a coding sequence derived from a particular APP gene which will result in observable clinical and/or behavioral symptoms, together with a measurable biochemical change in relevant brain structures can be determined empirically. Various changes in phenotype are of interest, and include, for example, progressive neurologic disease in the cortico-limbic areas of the brain expressed within a short period of the time from birth; increased levels of an APP gene or gene product above that of BACE1-knockout organisms and the development of a neurologic illness accompanied by premature death; gliosis and intracellular APP/A $\beta$  accretions present in the hippocampus and cerebral cortex; progressive neurologic disease characterized by diminished exploratory/locomotor behavior, impaired performance on memory and learning tests, and diminished 2-deoxyglucose uptake/utilization and hypertrophic gliosis in the cortico-limbic regions of the brain. Such phenotypic characteristics or changes thereof can be used to identify agents which are of interest for further study in the treatment of AD. Such changes can be measurably compared to BACE1-knockout mice as a standard or control organism.

The transgenic animals can also be studied using a species appropriate neurobehavioral test. For example, studies of locomotor/exploratory behavior in mice is a standard means of assessing the neuropsychology (File and Wardill, (1975) *Psychopharmacologia* (Berl) 44:53-59; Loggi *et al.*, (1991) *Pharmacol. Biochem. Behav.* 38:817-822). For example, for mice the "corner index" (CI), which is a quick and simple neurobehavioral test to screen animals for evidence of brain pathology, can be used. The CI in transgenic mice which express mutant and wild-type APP is also measured and can be compared to similar behavior in BACE1-knockout mice as a control. A low CI correlates with high mutant APP copy numbers, premature death, and neuropathologic findings. The CI exhibits a dosage dependent relationship to

APP copy number, which supports the validity of its use in assessing neurobehavioral signs in transgenic mice. The neuropathology of the animals also is evaluated. For rats, the Morris water maze test (see Morris, (1984) J. Neurosci. Meth. 11:47) can be used. A modified version of this test can be used with mice.

Brain regions known to be affected by the syndrome of interest are particularly reviewed for changes. When the disease of interest is Alzheimer's disease, the regions reviewed include the cortico-limbic region, including APP/A $\beta$  excretions, gliosis, changes in glucose uptake and utilization and A $\beta$  plaque formation. However, in strains of animals which are not long-lived, either naturally or when expressing high levels of APP, not all behavioral and/or pathological changes associated with a particular disease may be observed. As an example, transgenic FVB/N mice expressing high levels of APP tend not to develop detectable A $\beta$  plaques, whereas longer lived C57B6/ SJL F1 mice expressing identical transgenes do develop amyloid plaques which are readily detected with thioflavin S and Congo red. Immunologic studies of various brain regions also are used to detect transgene product. Comparing any of the foregoing with BACE1-knockout organisms can provide useful information in identifying novel therapeutic agents and diagnostics.

- The transgenic organisms (*e.g.*, BACE1 knockout organisms) of the invention can be used as controls for tester organisms for agents of interest, *e.g.* antioxidants such as Vitamin E or lazaroids, thought to confer protection against the development of AD. A test organism is treated with the agent of interest, and the neuropathology or behavioral pathology is compared to the BACE1-knockout organisms of the invention, wherein a neuropathology or behavior in the test animal treated with the agent of interest that is substantially similar to or superior to that of the BACE1-knockout organisms is an indication of protection from AD. The indices used preferably are those which can be detected in a live animal, such as changes in performance on learning and memory tests. The effectiveness can be confirmed by effects on pathological changes when the animal dies or is sacrificed.

Careful characterization of the transgenic animals of the invention should lead to elucidation of the pathogenesis of progressive neurologic syndromes such as AD. The sequence of molecular events in BACE1 metabolism leading to disease can be studied. In addition, understanding the role and activity of BACE1 homologues including, for example, BACE2, are provided by the transgenic organisms of the invention. The animals also are useful for studying various proposed mechanisms of pathogenesis, including horizontal transmission of disease. Such knowledge would lead to better forms of treatment for neurologic disorders.

The following examples are provided as a guide for those skilled in the art, and are not to be construed as limiting the invention in any way. All products are used according to manufacturer's instructions, and experiments are conducted under standard conditions, unless otherwise specified.

### EXAMPLE 1

#### Gene Targeting Vector and Embryonic Stem (ES) Cells

To examine the physiological roles of BACE1 and to determine whether BACE1 is the major  $\beta$ -secretase in neurons, mice with targeted inactivation of *BACE1* alleles were developed. A homologous recombination strategy in embryonic stem (ES) cells was used to inactivate the mouse *BACE1* gene. To target the BACE gene in ES cells, BACE genomic clones were isolated from a 129/Sv strain of mouse Lambda FIX II Library (Stratagene, CA) by using a partial mouse BACE cDNA containing the translation initiation codon as probe. In the *BACE1* targeting vector, a 2.0 kb *Bam*HI fragment containing the first coding exon which encode residues 1-87 (including the pro-peptide shown to be important for regulating BACE1 activity and flanking intronic sequences of the *BACE1* gene was replaced with a neomycin-resistance gene (Figure 1A) under the control of the PGK promoter. Introduction of a negative selection marker, the herpes simplex virus thymidine kinase gene, at the 5' end of the construct allowed the use of the positive and negative selection scheme.

The targeting vector was linearized at a unique *Not*I site before transfection into R1 ES cells, which were subjected to double selection. R1 ES cells were transfected

with the linearized *BACE1* targeting vector, and 2 clones (out of 112 screened) were targeted at the *BACE1* locus. Clones were picked and expanded, and DNA was isolated from a portion of the cells and screened by Southern blot analysis. Targeted cells were expanded and injected into C57BL/6J blastocysts to produce highly chimeric male mice that transmitted the targeted *BACE* allele in the germline. *BACE*<sup>+/-</sup> mice were intercrossed to obtain the *BACE*<sup>-/-</sup> animals. *BACE1*-targeted ES cells were used to generate the *BACE1*<sup>-/-</sup> mice. Genotype analyses of the *BACE1*<sup>-/-</sup> mice were performed by DNA blotting (Fig. 1B) and PCR methods (Figure 1C). Genotypes were determined by PCR amplification of tail or yolk sac DNA. The primer set (HC69: 5'-AGGCAGCTTTGTGGAGATGGTG (SEQ ID NO:1); HC70: 5'-CGGGAAATGGAAAGGCTACTCC (SEQ ID NO:2); and HC77: 5'-TGGATGTGGAATGTGTGCGAG (SEQ ID NO:3)) was used to detect the endogenous and targeted *BACE* alleles.

## EXAMPLE 2

### Antibody Preparation and Characterization

#### **Antibody Preparation**

A synthesized peptide corresponding to the C-terminal 12 residues of mouse BACE coupled to KLH was used to make the anti-peptide antibody (Research Genetics; Huntsville AL). To generate the HIS<sub>6</sub>-BACE fusion protein, a DNA fragment corresponding to residues 46 to 163 of BACE was subcloned into pTrcHisA (Invitrogen, San Diego, CA, see, also, GenBank Accession Nos. AF190725 and AAF04142; Vassar et al., Science 286:735, 1999, each of which is incorporated herein by reference). The HIS<sub>6</sub>-BACE fusion protein purified by Talon Metal Affinity Resin (Clontech; Palo Alto CA) chromatography was used as antigen for making the anti-fusion protein antibody (Covance Research Products Inc.; Denver PA). BACE anti-peptide and anti-fusion protein antibodies were generated in rabbits, and serum containing the antibodies was collected.

#### **Protein Extraction and Western Blot Analysis**

Mouse tissues were dissected out and homogenized in PBS in the presence of protease inhibitors. After extraction with 1% SDS, the lysates were centrifuged at 100,000xg

and supernatants were saved for western blot analyses. For western blot analysis, 50 µg protein was loaded in each lane, separated in a 10% Tris-Glycine gel, and transferred to PVDF membranes for immuno-detection with antibodies specific for BACE1 or superoxide dismutase (SOD1). A 1:3000 dilution of serum containing the anti-BACE antibodies was used as the primary antibody, and specific binding was detected using peroxidase-conjugated Protein A.

Western blot analysis confirmed that the targeting event (Example 1) led to inactivation of the *BACE1* gene. In *BACE1*<sup>+/-</sup> mice, BACE1 accumulated in brain to approximately 50% of the level of control littermate, whereas the brain of *BACE1*<sup>-/-</sup> mice showed no detectable level of BACE1 (Figure 1D). Similar results were observed using the antiserum specific to the carboxyl-terminal 13 residues of BACE1. These results confirm the inactivation of *BACE1*.

### EXAMPLE 3

#### **Generation of human APP and BACE recombinant adenoviruses**

A full-length human *BACE* cDNA was constructed from a near full-length clone isolated from a human fetal brain cDNA library (Origene Technologies Inc., MD) and a 5' cDNA encoding the N-terminal 41 amino acids of BACE obtained by RT-PCR of total RNA from HEK293 cells. Recombinant adenoviruses expressing wild type/mutant human APP or BACE were produced by cloning the full-length wild type/mutant human APP or BACE cDNA, respectively, into the pAd-Track-CMV shuttle vector. Under the control of distinct CMV promoters, this plasmid expresses the human APP or BACE, and in parallel, green fluorescent protein (GFP). The construct was integrated into the adenoviral backbone vector, pAd-Easy-1, by homologous recombination in *E. coli* strain BJ5183. The adenoviral construct was then cleaved with *PacI* and transfected in a packaging cell line (HEK 293 cells). The titer of the viral stocks was estimated based on the density of GFP-expressing cells.

**EXAMPLE 4****Primary cortical cultures and metabolic labeling**

Cortical neuronal cultures were established from brains of embryonic day 16.5 fetal mice. The dissected brain cortexes were suspended in HBSS supplemented with 0.25% trypsin and 0.01% DNase I and incubated at 37°C for 10 min. The tissues were then transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and dissociated by repeated trituration. The dispersed cells were collected by centrifugation and plated at approximately  $1 \times 10^6$  cells/well on 6 well cell culture plates (coated with poly-D-lysine) in B27/Neurobasal media (GIBCO/BRL; Gaithersburg MD). Neurons were allowed to mature for 4–7 days in culture before they were used for experiments. Primary neuronal cells cultured for 4 to 7 days were infected with  $5 \times 10^6$  plaque-forming units of adenovirus expressing human APP for 4 days in serum-free medium. For metabolic labeling, neuronal cells were pre-incubated for 30 min in methionine-free DMEM with 1% dialyzed bovine serum and then labeled with 700  $\mu\text{Ci/ml}$  of  $^{35}\text{S}$ -methionine in methionine-free medium for 5 hr.

For pulse-chase labeling, cells were pulsed for 45 min with methionine-free DMEM containing 1  $\text{mCi/ml}$   $^{35}\text{S}$ -methionine. Cells were then chased by washing and incubating in DMEM containing 1% dialyzed fetal bovine serum and 1 mM L-methionine at varying intervals, before the cells were lysed in immunoprecipitation buffer containing detergents and a protease inhibitor cocktail. After metabolic labeling, culture medium and cell extracts were immunoprecipitated and immunoprecipitates were fractionated on either 4%–20% Tris-glycine or 16% Tris-tricine SDS-PAGE. Gels were dried, exposed, and radioactive bands were quantified by phospho-imaging analysis.

To examine the effect of the absence of BACE1 on secretion of A $\beta$  peptides from neurons, primary cortical cultures from control, *BACE1*<sup>+/−</sup> and *BACE1*<sup>−/−</sup> embryos were derived from day 16.5 post coitum. The growth rate and morphology of the *BACE1*<sup>−/−</sup> cultures were identical to those of the *BACE1*<sup>+/−</sup> or control. Immunoprecipitation-mass spectrometry (IP-MS) analysis of conditioned culture media from control

neurons after 5 days in culture using an antisera (4G8) specific to epitopes between residues 17-28 of A $\beta$  revealed two prominent A $\beta$  species with mass values of 3171 and 4233 corresponding to mouse A $\beta$ 11-40 and A $\beta$ 1-40 respectively, in addition to several minor species including A $\beta$ 11-42 and A $\beta$ 1-42 (Figure 2B). While these A $\beta$  species are similarly observed in conditioned culture media from *BACE1*<sup>+/-</sup> neurons, secretion of these A $\beta$  species is abolished from *BACE1*<sup>-/-</sup> neurons except for the A $\beta$ 17-40 (p3) fragment (Figure 2B). These data establish that BACE1 is the major  $\beta$ -secretase required for cleavages of  $\beta$ APP at the +1 and +11 sites of A $\beta$  peptide in embryonic cortical neurons. Because a primary cleavage site for BACE2 is at +19/+20 of A $\beta$  and no A $\beta$ 20-40/42 or A $\beta$ 21-40/42 was detected, it was inferred therefore that BACE2 plays little role in the cleavage of APP in neurons.

To confirm the unique role of BACE1 in neurons, the processing of APP in control and *BACE1*<sup>-/-</sup> neuronal cultures following infection with a recombinant adenovirus expressing a humanized APP cDNA (a murine APP cDNA in which the A $\beta$ 1-42 region corresponds to the human A $\beta$ 1-42) bearing the Swedish variant (APP<sup>swe</sup>) was examined. Quantitative sandwich ELISA analyses of conditioned media from *BACE1*<sup>+/+</sup> cultures expressing APP<sup>swe</sup> showed high levels of A $\beta$ 1-40 and A $\beta$ 1-42 while undetectable levels of A $\beta$ 1-40 and A $\beta$ 1-42 were observed from media of *BACE1*<sup>-/-</sup> cultures expressing APP<sup>swe</sup> (Figure 2C). Metabolic labeling of control and *BACE1*<sup>-/-</sup> cortical neurons with <sup>35</sup>S-methionine for 5 hours and immunoprecipitation analysis using 4G8 antisera showed the presence of a major band (~4 kDa) corresponding to A $\beta$  and a minor band (~3.2 kDa) corresponding to p3 in control culture (Figure 2D), but, although p3 is readily secreted, no A $\beta$  accumulated in conditioned media from *BACE1*<sup>-/-</sup> cultures expressing APP<sup>swe</sup> (Figure 2D). Moreover, immunoprecipitation analysis using CT15, an antibody specific for the carboxyl-terminal 15 residues of APP<sup>12</sup>, revealed in *BACE1*<sup>-/-</sup> detergent lysates the accumulation of full length APP as well as APP  $\beta$ -CTF (Figure 2E); however, this approach failed to detect APP  $\beta$ -CTF in the lysates, which are in control lysates (Figure 2E). Taken together, these results confirm that BACE1 is the primary  $\beta$ -secretase in cortical neurons and infer that BACE2 does not play a significant role in the processing of APP in neuronal cultures.

Since  $\alpha$ -secretases and  $\beta$ -secretases compete for the same substrate, we anticipated that in the absence of BACE, APP derivatives produced by the action of  $\alpha$ -secretase might be increased. To determine whether the rate of secretion of  $\alpha$ -secretase derived APP soluble ectodomain (APPs $\alpha$ ) is altered, the processing of APPswe in *BACE1*<sup>+/+</sup> and *BACE1*<sup>-/-</sup> cortical neurons was examined. Pulse-chase studies revealed that there is an increase in the rate of secretion of APPs $\alpha$  in *BACE1*<sup>-/-</sup> neurons as compared to controls (Figures 3C-3E). Furthermore, no accumulation of either  $\beta$ -CTF or A $\beta$  in the *BACE1*<sup>-/-</sup> neuronal cultures was detected (Figures 3A and 3B). These results establish that BACE1 competes with  $\alpha$ -secretase in APP processing and further confirm the view that BACE1 is the major  $\beta$ -secretase in neurons.

### Example 5

#### Mass Spectrometric Analysis

The  $\beta$ -amyloid peptides were captured with 4G8 monoclonal antibody (Senetek; Napa CA) by immunoprecipitation from conditioned media of cultured neurons. After final wash, the immunoprecipitates were rinsed twice with 5 mM HEPES buffer (pH 7.0). A 1  $\mu$ l sample was spotted on NP-1 series ProteinChip<sup>TM</sup> array and analyzed by surface-enhanced laser desorption/ionization time of flight MS (Ciphergen Biosystems, Palo Alto CA) in the presence of CHCA matrix solution (Ciphergen Biosystems). External standards were used for calibration.

### EXAMPLE 6

#### Determination of A $\beta$ 1-42/43 and A $\beta$ 1-40 Levels

Two-site ELISA's that specifically detect the C-terminus of A $\beta$  were performed to measure A $\beta$  levels as suggested by the manufacturer (Biosource International; Camarillo CA). Culture media of neuronal cells infected with adenovirus expressing human APP were collected and analyzed using the quantitative sandwich ELISA to determine both A $\beta$ 1-42 and A $\beta$ 1-40 levels.

To confirm that BACE1 cleaves APP at both the +1 and +11 sites of A $\beta$ , neuronal cultures infected with adenovirus expressing either humanized wild type APP

(hAPPwt) or its variants (hAPPswe or hAPP717) were examined and the secretion of A $\beta$  peptides from conditioned media as well as the accumulation of both +1 and +11 derived  $\beta$ -CTFs from cell lysates measured. As expected, IP-MS analysis of conditioned media using 4G8 antibody showed that the human and murine A $\beta$ 1-40 and A $\beta$ 1-42 were secreted, however, the human A $\beta$ 11-40 peptide was not secreted into culture media from murine primary neurons infected with adenovirus expressing hAPPwt, although the murine A $\beta$ 11-40 was readily detected. Similar results were also observed with murine neurons infected with adenovirus expressing hAPPswe or hAPP717. This apparent discrepancy raised the possibility that the cleavage site at +11 of A $\beta$  is species-specific, *i.e.*, human or murine BACE1 cleaves respectively, human or murine APP at +11 site of A $\beta$  whereas no species selectivity occurs at the +1 site. To test this possibility, the processing of human APP by co-infecting murine neuronal cultures with adenovirus expressing both human BACE1 and hAPPwt or its variants was examined. IP-MS analysis of conditioned media using 4G8 antibody now revealed the secretion of human A $\beta$ 11-40 peptide in addition to the murine A $\beta$ 11-40 peptide from murine neurons co-expressing human BACE1 and hAPPwt. The human A $\beta$ 11-40 peptides are also secreted by primary neurons co-expressing human BACE1 and hAPPswe or hAPP717.

In addition, since human BACE1 cleaves human APP at the +11 site of A $\beta$ , the +11 derived  $\beta$ -CTF was examined to determine whether it accumulated in lysates of neurons co-expressing human BACE1 and human APPwt or its variants. As expected, while  $\beta$ -CTFs are readily immunoprecipitated using the CT15 antibody from control, hAPPwt, hAPPswe or hAPP717 lysates, the +1 derived  $\beta$ -CTF is observed only in the hAPPswe lysate. However, when neurons co-expressing human BACE1 and hAPPwt or hAPPswe or hAPP717 there is secreted a peptide corresponding to the +11 derived  $\beta$ -CTF (+11-CTF) in addition to the +1 derived  $\beta$ -CTF. Taken together, these results support the view that the cleavage site at +11 of A $\beta$  is species-specific. To begin to access the determinants that govern this selectivity, the amino acid sequences of A $\beta$  between humans and mice were compared; there is a sequence divergence around the +11 site whereas there is absolute conservation at the +1 site of A $\beta$  (see Figure 2A). Mutagenesis studies will

allow determination of the amino acid residue(s) that confer species specificity at the +11 site of A $\beta$ . Although A $\beta$ 11-40/42 peptides has been previously observed in neuronal cultures as well as in the brains of cases of AD, the roles of these peptides in the pathogenesis of AD was not understood. A $\beta$  beginning at +11 is a major species in rodents *in vivo* and this peptide is more fibrillogenic and neurotoxic than full length A $\beta$  *in vitro*. Because the finding that the +11 site is a major cleavage site for BACE1, the involvement of A $\beta$ 11-40/42 in pathogenesis of AD is important. A $\beta$ 11-40/42 plays a critical role in AD, thus antibodies specific to A $\beta$ 11-40/42 would prove useful for diagnoses of sporadic AD. The demonstration that the cleavage at +11 is species-specific would infer that the published mutant human APP transgenic models would not be expected to secrete the human A $\beta$ 11-40/42 (because murine BACE1 does not cleave at the +11 site) and transgenic mice over-expressing either murine wild type APP or its variants may be instructive in clarifying the pathogenic roles of A $\beta$ 11-40/42.

The secretion of A $\beta$  peptides (A $\beta$ 1-40/42 as well as A $\beta$ 11-40/42) from neurons is abolished in cultures of BACE1-deficient embryonic cortical neurons derived from BACE1- knockout mice. Moreover, while the intracellular  $\beta$ -carboxy terminal fragments of  $\beta$ APP ( $\beta$ -CTFs) and the corresponding APP $\alpha$  fragments are not generated in *BACE*<sup>-/-</sup> neurons, the rate of APP $\alpha$  secretion is increased in *BACE*<sup>-/-</sup> neurons as compared to controls. These results establish that BACE1 is the principal neuronal protease required to cleave  $\beta$ -amyloid precursor protein (APP) at +1 and +11 sites of A $\beta$  that generate N-termini of A $\beta$ . In addition, the invention provides for the first time, that while both human and murine BACE1 are capable of cleaving either human or murine  $\beta$ APP at the +1 site of A $\beta$ , the cleavage at the +11 site is species-specific. Taken together, these results have important implications for the development of novel therapeutic strategies in Alzheimer's disease.

While both  $\alpha$ -secretase and  $\beta$ -secretase activities represent therapeutic targets for the development of novel protease inhibitors for AD, the discovery of BACE1 and BACE2 now provides the opportunity to determine whether these aspartic proteases are indeed high priority targets. The demonstration that BACE1 is the major

$\beta$ -secretase in neurons provides excellent rationale for focusing on the design of novel therapeutics to inhibit BACE1 activity in brain as well as using A $\beta$ 11-40//42 as novel tools for diagnosing AD. The transgenic organisms of the invention allow for the identification of other important substrates for BACE1 and the evaluation of BACE1 knockout. This information will have significant impact in the design of specific drugs to inhibit BACE1 in the central nervous system. To illustrate this principle, it is instructive to consider the emerging view that the presenilins (PS1 and PS2), which when mutated cause familial AD and which are important for the intramembranous proteolysis of several proteins, including APP and Notch1, may be the putative  $\gamma$ -secretase. Presenilins are involved in the proteolytic processing of Notch1 and are critical for Notch1 functions. PS1 null mice, which die before or at birth, have a developmental defect in patterning of somites; a phenotype resembling that observed in the Notch1 null mice. Recent demonstrations that PS1 co-fractionates with  $\gamma$ -secretase activity, that transition-state analogue inhibitors of  $\gamma$ -secretase can covalently label PS, and that two transmembrane aspartates are required for  $\gamma$ -secretase activity provide support for the view that PS1/2 may possess  $\gamma$ -secretase activity or is a co-factor intimately associated with  $\gamma$ -secretase cleavages. Alternatively, PS1/2 may play a role in trafficking of APP or other molecules. Consistent with the idea that  $\gamma$ -secretase activity is subserved by a multi-subunit catalytic complex is the recent identification of the type 1 transmembrane protein, nicastrin, which interacts with presenilins that are known to modulate both  $\gamma$ -secretase activity and Notch1 function. Thus, the design of therapeutics that inhibit  $\gamma$ -secretase and thus influence Notch1 processing could have in the adult, impact on some cell populations (hematopoietic cell) that utilize Notch1 signaling for cell fate decision. In this case, it would be necessary to try to develop highly selective inhibitors that act principally on  $\gamma$ -secretase activities that cleave APP and have less inhibiting potency on Notch1 cleavage.

The demonstration that BACE1 null mice are viable allows for the development of inhibitors that are brain penetrate (*i.e.*, can cross the blood brain barrier), bind to the active sites (extracellular) of BACE1 to ameliorate  $\beta$ -amyloid deposition, and are without profound adverse effects. BACE1 null mice are valuable for testing whether

the  $\beta$ -amyloid burden can be reduced in mutant APP transgenic models lacking BACE1. Such an outcome would greatly encourage investigators to design novel drugs to inhibit BACE1 activity. The recent report documenting the crystal structure of the protease domain of BACE1 associated with an eight-residue inhibitor provide valuable information towards the development of specific drugs to inhibit BACE1 activity. These compounds can be tested in transgenic mice to determine whether they ameliorate A $\beta$  deposition. If so, these therapeutic can be brought rapidly into clinical trials.

#### **EXAMPLE 7**

##### **BACE1 EXPRESSION IN BRAIN AND CORRELATION TO AMYLOIDOSIS**

To begin to assess the role of BACE1 as a determinant of selective vulnerability of the brain to A $\beta$  amyloidogenesis, the level and distribution of BACE1 in the CNS and various other tissues was examined in BACE1 knockout mice and wild type littermates. In contrast to the ubiquitous expression of BACE1 mRNA in a variety of tissues, BACE1 protein was abundantly expressed only in the brain and was undetectable in other non-neural tissues, including pancreas, heart, liver and kidney. BACE1 expression also was examined in various regions of the CNS, including the frontal cortex, posterior cortex, cerebellum, hippocampus, olfactory bulb, striatum, thalamus, midbrain, entorhinal cortex, pons, medulla and spinal cord of new born mice (new born mouse brain blot; Chemicon). The accumulation of BACE1 across various regions of the brain was uniform when normalized to the level of  $\beta$ 3 tubulin, except that a relatively higher level was observed in the olfactory bulb and a relatively lower level was observed in the frontal cortex and spinal cord.

Although BACE1 protein was expressed at comparable level in most brain regions as shown by western blot, BACE1-specific immunoreactivities were particularly localized in the hippocampus, a region that is critical for learning and memory and is particularly vulnerable in AD. Strong expression was observed in the hilus of dentate gyrus and stratum lucidum of CA3 region (terminal field of mossy fiber pathway); no specific expression was observed in brain sections prepared from BACE1 knockout mice.

To begin to assess whether the staining pattern observed for BACE1 is pre-synaptic, the staining patterns of several markers were examined. Littermate control and BACE1 knockout mice (or APP and PS1 double transgenic mice, see below) were sacrificed and perfused with 4% PFA, then brain and other organs were paraffin embedded, sectioned, and processed for immunohistochemical analysis using the peroxidase-anti-peroxidase method. Antibodies included those specific for BACE1, synaptophysin, syntaxin, and MAP2, and were used in conjunction with hematoxylin and eosin staining. For BACE1 immunohistochemical analysis, an IgG purified anti-BACE1 fusion protein antibody was applied (1  $\mu$ g/ml) as primary antibody in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 1% Triton X-100 detergent, after retrieving the antigen by heating the tissue section in a microwave oven.

The BACE1 staining pattern in the hippocampus was remarkably similar to two well characterized pre-synaptic terminal markers, synaptophysin and syntaxin, and was distinct from that of MAP2, which is a postsynaptic marker. Moreover, under higher magnification, BACE1 immunoreactivities were localized to the giant boutons of the mossy fibers that form synapses with the hilar mossy cells and proximal dendrites of CA3 pyramidal cells; the giant boutons also were readily labeled by anti-synaptophysin antisera. These results indicate that BACE1 protein expression is localized to pre-synaptic terminals. Because hippocampal granule cells are continuously undergoing turnover throughout the life of the animal, the particular enrichment of BACE1 protein in these highly plastic cells further indicates that BACE1 can have a role in either synaptic development or plasticity in the brain.

The role of BACE1 in astrocytes was examined with respect to the processing of APP in *BACE1*<sup>+/+</sup>, *BACE1*<sup>+/-</sup> and *BACE1*<sup>-/-</sup> astrocyte cultures four days after infection with a recombinant adenovirus expressing a humanized APP cDNA bearing the Swedish variant (APP<sup>swe</sup>). *BACE1*<sup>+/+</sup>, *BACE1*<sup>+/-</sup> and *BACE1*<sup>-/-</sup> neuronal cultures were labeled for 5 hours with <sup>35</sup>S-methionine, then full length APP and APP-CTF's were immunoprecipitated with CT15. The signal intensity of APP  $\beta$ -CTF and  $\alpha$ -CTF was quantified by phospho-imaging and was normalized against APP full length protein.

Protein blot analysis of *BACE1*<sup>-/-</sup> detergent lysates revealed the accumulation of full length APP as well as APP  $\alpha$ -CTF; APP  $\beta$ -CTF was not detected. To examine the effect of the absence of BACE1 on secretion of A $\beta$  peptides from astrocytes, conditioned media derived from *BACE1*<sup>-/-</sup> cultures were subjected to ELISA and mass spectrometric analyses. A $\beta$ 1-40 and A $\beta$ 1-42 were easily detected from *BACE1*<sup>+/+</sup> and *BACE1*<sup>+/-</sup> astrocyte cultures by A $\beta$  ELISA assay, but were not detectable from *BACE1*<sup>-/-</sup> cultures. APP  $\beta$ -CTF from *BACE1*<sup>+/-</sup> cultures was reduced compared to *BACE1* wild type cultures (n=3; p<0.04, Student's t-test). APP  $\beta$ -CTF was barely detectable in *BACE1*<sup>+/-</sup> astrocyte cultures infected with the APPswe adenovirus. Quantitative sandwich ELISA analysis of conditioned medium from *BACE1*<sup>+/-</sup> astrocyte cultures expressing APPswe showed an approximately 50% reduction of levels of A $\beta$ 1-40 and A $\beta$ 1-42 compared to *BACE1*<sup>+/+</sup> cultures.

Mass spectrometric profiles of secreted human A $\beta$  1-19, 1-20, 1-40, and 1-42 from conditioned media of cortical neurons or glial cells infected with adenovirus expressing APPswe were determined by using PS1 CIPHERGEN ProteinChip™ system coated with 6E10, a monoclonal antibody specific against human A $\beta$  1-16 revealed identical results as were observed by ELISA. Furthermore, BACE1 deficient astrocytes co-infected with adenoviruses expressing both APPswe and BACE1 restored the ability to secrete A $\beta$  peptides into culture medium. The cultured astrocytes showed stronger BACE2 and  $\alpha$ -secretase activities than the cultured neurons (n=4; p<0.001, Student's t-test). These results demonstrate that BACE1, in addition to being the principal  $\beta$ -secretase in neurons, also is the principal  $\beta$ -secretase in cortical astrocytes.

Besides BACE1 and  $\gamma$ -secretase, APP can also be cleaved within the A $\beta$  region by putative " $\alpha$ -secretases" such as TACE and ADAM10 at residue +16, or by BACE2 at residues +19 and +20 of A $\beta$ . Since these three cleavages occur within the A $\beta$  domain, action by BACE2 and  $\alpha$ -secretase would preclude the formation of toxic A $\beta$  peptide. In contrast to BACE1, BACE2, TACE and ADAM10 mRNA levels are

relatively low in brain. However, it is not known whether the lower expression level of BACE2 and TACE/ADAM10 correlates with lower  $\alpha$ -secretases and BACE2 mediated anti-amyloidogenic activities in neurons.

Using the CIPHERGEN PS1 ProteinChip™ array coated with 6E10 antibody, the relative amount of BACE2 derived A $\beta$ 1-19 and A $\beta$ 1-20 and  $\alpha$ -secretase derived A $\beta$ 1-15 or A $\beta$ 1-16 fragments were examined, and normalized to BACE1 derived A $\beta$ 1-40. BACE2 mediated cleavages (at +19 and +20 of A $\beta$ ) were much higher in cultured astrocytes as compared to neurons (see Figure 4). Similar studies in cultured cell lines, such as fibroblasts and COS-1 cells also revealed high levels of BACE2 or  $\alpha$ -secretase activities coupled with low level of BACE1 activity. Because the absence of BACE1 abolished secretion of BACE2 derived A $\beta$ 1-19 and A $\beta$ 1-20 peptides, BACE2 primarily cleaves APP at +19 and +20 sites, but not at +1 site of A $\beta$ . These results confirm that the high level of BACE1 coupled with low levels of BACE2 and  $\alpha$ -secretase activities in neurons predispose these cells to A $\beta$  amyloidogenesis.

To begin to examine whether BACE1 is a determinant of selective vulnerability of neurons to amyloidogenesis, the relative levels of BACE1 protein or activity in cultured cortical astrocytes as compared to neurons was examined. Protein blot analysis revealed that BACE1 protein level in astrocytes was much lower than that of neurons. This result demonstrates that neurons are the primary source of A $\beta$  and that BACE1 is a major susceptibility factor that predisposes neurons to A $\beta$  amyloidosis in the brain. These results were extended by examining A $\beta$  generation and deposition in a mouse model of amyloidosis lacking BACE1. Since the above results demonstrated that BACE1 was required for the secretion of A $\beta$  peptides by neurons, it was expected that A $\beta$  formation and deposition would be abolished in APP mutant mice lacking BACE1. However, because the absence of BACE1 led to an increase in the  $\alpha$ -secretase derived p3 peptide, it also was plausible that p3 could contribute to A $\beta$  deposition in brains of APP mice lacking BACE1. To test this possibility and to determine whether partial reduction of BACE1 ameliorate A $\beta$  deposition, APP/PS1 mutant mice, which express reduced levels of BACE1, were generated. The absence of BACE1 abolished the formation of A $\beta$ 1-40 and A $\beta$ 1-42 as determined by standard

ELISA methods. Moreover, whereas abundant A $\beta$  deposition was observed in APP/PS1 double transgenic mice, no A $\beta$  plaques were detected in APP/PS1 mice lacking BACE1. BACE1 immunoreactivity also was observed in dystrophic neurites surrounding amyloid plaques in the brains of APP/PS1 double transgenic mice.

Together with the cell culture studies described above, the present results establish that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of A $\beta$  in the central nervous system and indicate the potential therapeutic value of inhibiting BACE1 in efforts to ameliorate A $\beta$  deposition in AD. These results also demonstrate that the anti-BACE1 antibodies are specifically reactive with BACE1, and that the antibodies can be used as a diagnostic reagent to identify regions of selective vulnerability of brain amyloidosis in Alzheimer's disease.

Although both BACE1 and BACE2 are expressed ubiquitously, BACE1 mRNA levels are particularly high in brain and pancreas, whereas the levels of BACE2 mRNA are relatively low in all tissues, except in brain where it is nearly undetectable. While BACE1 was shown to be the principal  $\beta$ -secretase necessary to cleave APP to generate A $\beta$  *in vivo*, *in vitro* studies indicated that BACE2 was capable of cleaving APP more efficiently at sites within the A $\beta$  domain as compared to the +1 site of A $\beta$ . As BACE1 is the principal  $\beta$ -secretase in neurons and BACE2 serves to limit the secretion of A $\beta$  peptides, the present results indicate that BACE1 is a pro-amyloidogenic enzyme, while BACE2 is an anti-amyloidogenic protease. In this scenario, the relative levels of BACE1 and BACE2 in neurons are determinants of A $\beta$  amyloidosis, and the secretion of A $\beta$  peptides would be expected to be the highest in neurons/brain as compared to other cell types/organs because neurons express high levels of BACE1 coupled with low expression of BACE2. If a high level of BACE1 coupled to a low level of BACE2 is a critical factor that selectively predisposes the brain to A $\beta$  amyloidosis, AD would be predicted to involve the brain rather than heart or pancreas. Seemingly inconsistent with this hypothesis is that very high level of BACE1 mRNA expression is observed in the pancreas. However, some of the pancreatic mRNAs appear to be alternatively spliced to generate a BACE1 isoform that is incapable of cleaving APP Bodendorf et al., J. Biol. Chem. 276:12019-12023,

2001). Thus, our results now demonstrating that while neurons in the brain possess high level of BACE1 coupled with low level of BACE2 activity, astrocytes or fibroblasts showed low level of BACE1 and high BACE2 activity, supports this hypothesis. Importantly, to test in vivo the hypothesis that the abundance of BACE1 is a major determinant of selective vulnerability of neurons to A $\beta$  amyloidosis, we took a genetic approach to reduce the level of BACE1 selectively in neurons/brain of mutant APP/PS1 transgenic mice. As disclosed herein, while the deletion of BACE1 abolished the secretion and deposition of A $\beta$ , the partial reduction of BACE1 (to 50% of normal level) significantly ameliorated amyloid plaque deposition in a mouse model of A $\beta$  amyloidosis. These results demonstrate that BACE1 is a major determinant of selective vulnerability of neurons to the extracellular deposition of A $\beta$  in the central nervous system and indicate the potential importance of polymorphisms that can act, for example, to increase levels of BACE1 and thereby predispose individuals to AD.

Although the invention has been described with reference to the certain embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed:

1. A method for modulating the production of A $\beta$ 11-40/42 peptide fragments comprising contacting a sample or cell containing a beta-site APP-cleaving enzyme 1 (BACE1) and an amyloid precursor protein (APP) with a BACE1-modulating agent such that production of A $\beta$ 11-40/42 is modulated.
2. The method of claim 1, wherein the modulation is inhibition of A $\beta$ 11-40/42 peptide formation.
3. The method of claim 1, wherein the contacting is *in vivo*.
4. The method of claim 1, wherein the contacting is *in vitro*.
5. The method of claim 1 wherein the BACE1-modulating agent is an anti-BACE1 antibody or a BACE1 antisense molecule.
6. A method for identifying a compound which inhibits beta-site APP-cleaving enzyme 1 (BACE1) expression or activity comprising:
  - a) incubating components comprising the compound, BACE1 polynucleotide or polypeptide, and an amyloid precursor protein (APP) under conditions sufficient to allow the components to interact; and
  - b) measuring the production of a BACE1 specific enzymatic product.
7. The method of claim 6, wherein the compound is a peptide.
8. The method of claim 6, wherein the compound is a small molecule inhibitor.
9. The method of claim 6, wherein the BACE1 polynucleotide or polypeptide is expressed in a cell.

10. The method of claim 6, wherein the BACE1 specific enzymatic product includes a sequence of A $\beta$ 11-40/42.
11. A compound identified by the method of claim 6.
12. The compound of claim 11, in a pharmaceutically acceptable carrier.
13. A method for diagnosing a subject having or at risk of having an A $\beta$ 11-40/42 peptide accumulation disease, the method comprising:
  - measuring the amount of beta-site APP-cleaving enzyme 1 (BACE1) in a biological sample from the subject; and
  - comparing the amount BACE1 with a normal standard value of BACE1, wherein a difference between the measured amount and the normal sample or standard value provides an indication of the diagnosis of A $\beta$ 11-40/42.
14. The method of claim 13, wherein the biological sample is blood, serum, cerebrospinal fluid or central nervous system (CNS) tissue.
15. The method of claim 13, wherein the difference is an increase in BACE1.
16. The method of claim 13, wherein the amount BACE1 is measured by detecting the amount of a polynucleotide encoding BACE1.
17. The method of claim 16, wherein the polynucleotide is mRNA.
18. The method of claim 17, wherein the mRNA is detected by PCR.
19. The method of claim 13, wherein the amount of BACE1 is detected by contacting the sample with an agent that specifically binds to a BACE1 polypeptide.
20. The method of claim 19, wherein the agent is an antibody.

21. The method of claim 20, wherein the antibody is a monoclonal antibody.
22. The method of claim 20, wherein the antibody is a polyclonal antibody.
23. The method of claim 19, wherein the A $\beta$ 11-40/42 accumulation disease is Alzheimer's disease.
24. The method of claim 13, further comprising detecting the level of an APP fragment, wherein an increase in the presence of the fragment is indicative of Alzheimer's disease.
25. The method of claim 24, wherein the APP fragment is an A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ 11-40, or A $\beta$ 11-42 fragment.
26. The method of claim 25, wherein the fragments are detected by contacting the sample with an agent the specifically binds to an A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ 11-40, or A $\beta$ 11-42 fragment.
27. The method of claim 26, wherein the agent is an antibody.
28. The method of claim 20 or 27, wherein the antibody is detectably labeled.
29. The method of claim 28, wherein the detectable label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

30. A method for diagnosing a subject having or at risk of having Alzheimer's disease, the method comprising:

measuring A $\beta$ 11-40/42 in a biological sample from the subject; and  
comparing the amount of A $\beta$ 11-40/42 with a normal sample or standard value of A $\beta$ 11-40/42, wherein a difference between the amount in the normal sample or standard value is indicative of a subject having or at risk of having Alzheimer's disease.

31. The method of claim 30, wherein the biological sample is cerebrospinal fluid, central nervous system (CNS) tissue, serum or blood.

32. The method of claim 30, wherein the difference is an increase in A $\beta$ 11-40/42 and the increase is indicative of a disposition for Alzheimer's disease.

33. The method of claim 30, wherein the difference is a decrease in A $\beta$ 11-40/42.

34. The method of claim 30, wherein the amount of A $\beta$ 11-40/42 is detected by contacting the sample with an agent that specifically binds to A $\beta$ 11-40/42.

35. The method of claim 34, wherein the agent is an antibody.

36. The method of claim 35, wherein the antibody is a monoclonal antibody.

37. The method of claim 35, wherein the antibody is a polyclonal antibody.

38. The method of claim 34, wherein the agent is an antibody fragment.

39. The method of claim 30, further comprising detecting the level of a BACE1 polypeptide or polynucleotide, wherein an increase in the level of BACE1 is indicative of Alzheimer's disease.

40. The method of claim 35, wherein the antibody is detectably labeled.

41. The method of claim 40, wherein the detectable label is selected from the group consisting of a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

42. A transgenic non-human animal having a transgene disrupting expression of BACE1, chromsomally integrated into the germ cells of the animal, and have a phenotype of reduced A $\beta$  peptide as compared with a wild-type animal.

43. The transgenic non-human animal of claim 42, wherein the animal is an avian, bovine, ovine, piscine, murine, or porcine species.

44. The transgenic non-human animal of claim 42, wherein the animal is heterozygous or homozygous for the disruption.

45. The transgenic non-human animal of claim 42, wherein the transgene comprises a BACE1 antisense polynucleotide.

46. A method for producing a transgenic non-human animal having a phenotype characterized by reduced expression of BACE1 polypeptide, the method comprising:

- (a) introducing at least one transgene into a zygote of an animal, the transgene(s) comprising a DNA construct encoding a selectable marker,
- (b) transplanting the zygote into a pseudopregnant animal,
- (c) allowing the zygote to develop to term, and
- (d) identifying at least one transgenic offspring whose genome comprises a disruption of the endogenous BACE1 polynucleotide sequence by the transgene.

47. The method of claim 46, wherein the introducing of the transgene into the embryo is by introducing an embryonic stem cell containing the transgene into the embryo.

48. The method of claim 46, wherein the transgenic non-human animal is heterozygous or homozygous for the disruption.

49. The method of claim 46, wherein the introducing of the transgene into the embryo is by infecting the embryo with a retrovirus containing the transgene.

50. A method for identifying an agent that modulates the expression or activity of BACE1, said method comprising:

administering an agent to be tested to an organism; and  
comparing the phenotype of the organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent, whereby a phenotype substantially equal to the BACE1-knockout organism is indicative of an agent that modulates BACE1 expression or activity.

51. The method of claim 50, wherein the organism is a transgenic organism.

52. The method of claim 51, wherein the transgenic organism is transgenic for overexpression of BACE1; APP expression; A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ 11-40, A $\beta$ 11-42 expression; or a combination thereof.

53. The method of claim 50, wherein the expression of BACE1 is detected by measuring the amount of BACE1 polynucleotide in the organism.

54. The method of claim 53, wherein the BACE1 polynucleotide is RNA or DNA.

55. The method of claim 54, wherein the RNA is mRNA.

56. The method of claim 50, wherein the activity of BACE1 is detected by measuring BACE1 cleavage of APP.

57. The method of claim 50, wherein the phenotype of the organism is associated with Alzheimer's disease.

58. The method of claim 57, wherein the phenotype associated with Alzheimer's disease is characterized as having a phenotype of impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

59. A method for screening for an agent, which ameliorates symptoms of Alzheimer's disease, said method comprising comparing an effect of an agent on an organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent,

wherein the organism has a phenotype associated with Alzheimer's disease, and wherein an agent which ameliorates said phenotype is identified by having a substantially equal or superior phenotype of the organism in comparison with the BACE1-knockout organism.

60. The method of claim 59, wherein the phenotype of the organism is characterized as having a phenotype of impaired performance on memory learning tests and abnormal neuropathology in a cortico-limbic region of the brain.

61. The method of claim 59, wherein the organism is a transgenic organism.

62. The method of claim 59, wherein the phenotype is measured by assessing an organism's performance on memory and learning tests.

63. The method of claim 59, wherein the phenotype is measured by assessing the neuropathology in a cortico-limbic region of the brain.

64. A method for screening for an agent, which ameliorates symptoms of Alzheimer's disease, said method comprising comparing an effect of an agent on a transgenic organism contacted with the agent with that of a BACE1-knockout organism not contacted with the agent,

wherein the transgenic organism is characterized as having a phenotype of impaired performance on memory learning tests or abnormal neuropathology in a cortico-limbic region of the brain and the BACE1-knockout organism has a phenotype of reduced expression of BACE1, and

wherein the impaired performance and the abnormal neuropathology are in compared with the BACE1-knockout organism,

whereby an agent which ameliorates the symptoms is identified by substantially equal or superior performance of the transgenic organism as compared with the BACE1-knockout organism on the memory and learning tests.

65. A kit useful for the detection of an A $\beta$ 11-40/42 accumulation disorder comprising carrier means containing therein one or more containers wherein a first container contains a nucleic acid probe that hybridizes to a nucleic acid sequence BACE1 or an antibody probe specific for BACE1 or A $\beta$ 11-40/42.

66. The kit of claim 65, wherein the probe comprises a detectable label.

67. The kit of claim 65, wherein the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

68. A method for predicting the therapeutic effectiveness of a compound for treating Alzheimer's disease in a subject comprising measuring the accumulation of AB11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide before and after treatment with the compound,

wherein a decrease in accumulation of peptide fragments or a decrease in the level of BACE1 polynucleotide or polypeptide after treatment is indicative of a compound that is effective in treating the disease.

69. A method for monitoring the progression of Alzheimer's disease comprising measuring the accumulation of AB11-40/42 peptide fragments in the subject or the level of BACE1 polynucleotide or polypeptide at a first time point and a second time point, thereby monitoring the progression of the disease.

70. An substantially purified antibody that specifically binds a beta-site APP-cleaving enzyme 1 (BACE1) polypeptide or an epitopic determinant thereof.

71. The antibody of claim 70, which is present in an antiserum.

72. The antibody of claim 70, which comprises polyclonal antibodies.

73. The antibody of claim 70, which is a monoclonal antibody.

74. The antibody of claim 70, wherein the epitopic determinant comprises amino acid residues 46 to 164 of BACE1.

75. A method of detecting a beta-site APP-cleaving enzyme 1 (BACE1) polypeptide in a sample, the method comprising contacting the sample with the antibody of claim 70 under conditions that allow specific binding of the antibody to BACE1 or an epitopic determinant thereof, and detecting specific binding of the antibody to a component of the sample.

76. The method of claim 75, wherein the sample is a tissue sample, which is obtained from a subject.

77. The method of claim 76, wherein the tissue sample is a brain tissue sample.

78. The method of claim 76, wherein the subject has or is suspected of having a disorder associated with an accumulation of amyloid plaques.

79. The method of claim 78, wherein the disorder is Alzheimer's disease.

80. The method of claim 75, wherein the antibody comprises a detectable label, and wherein said detecting specific binding comprises detecting the label.

81. The method of claim 75, further comprising contacting the sample with a reagent that specifically binds the antibody, wherein detecting specific binding of the antibody comprises detecting specific binding of the reagent.

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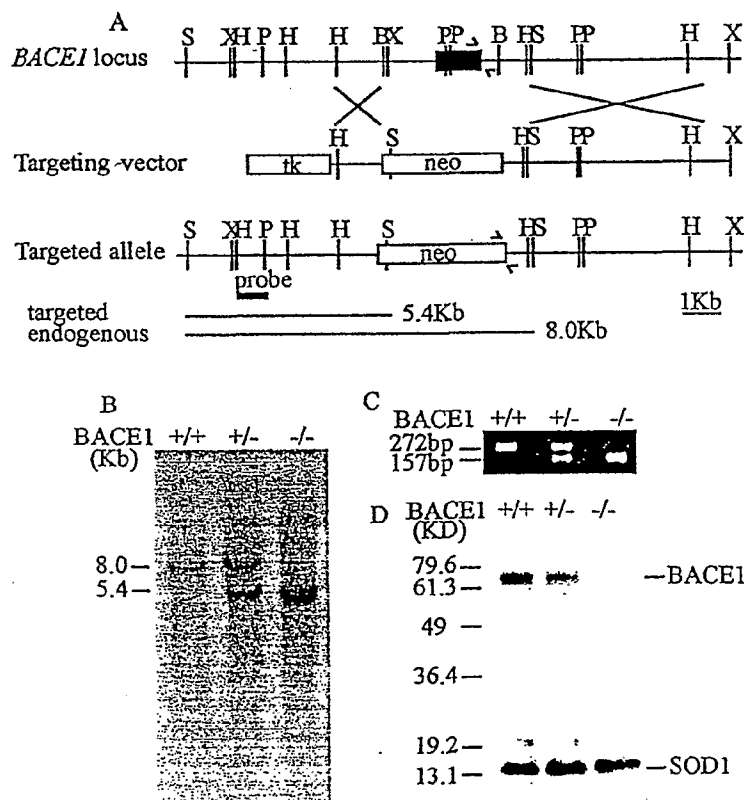


FIGURE 1

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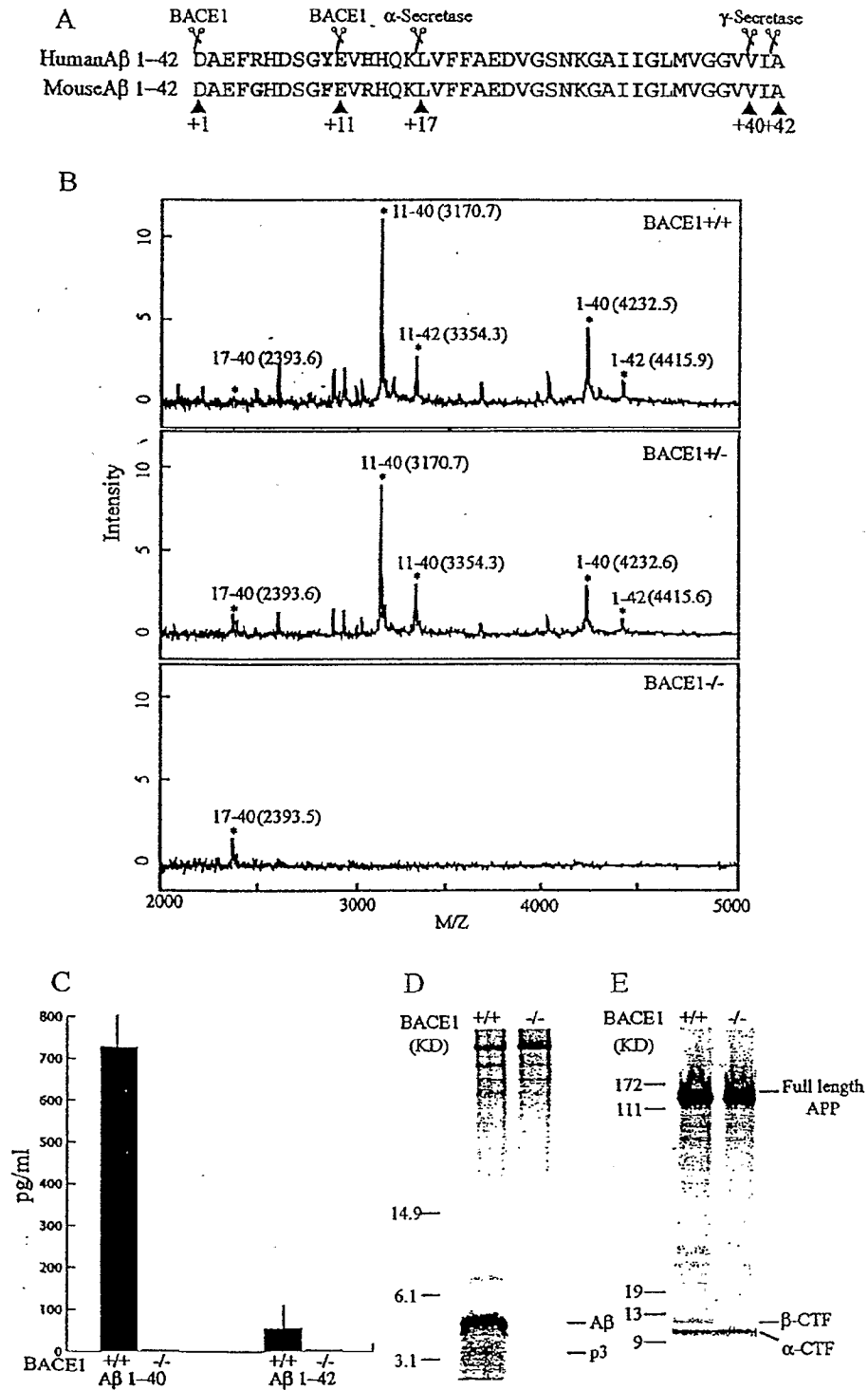


FIGURE 2

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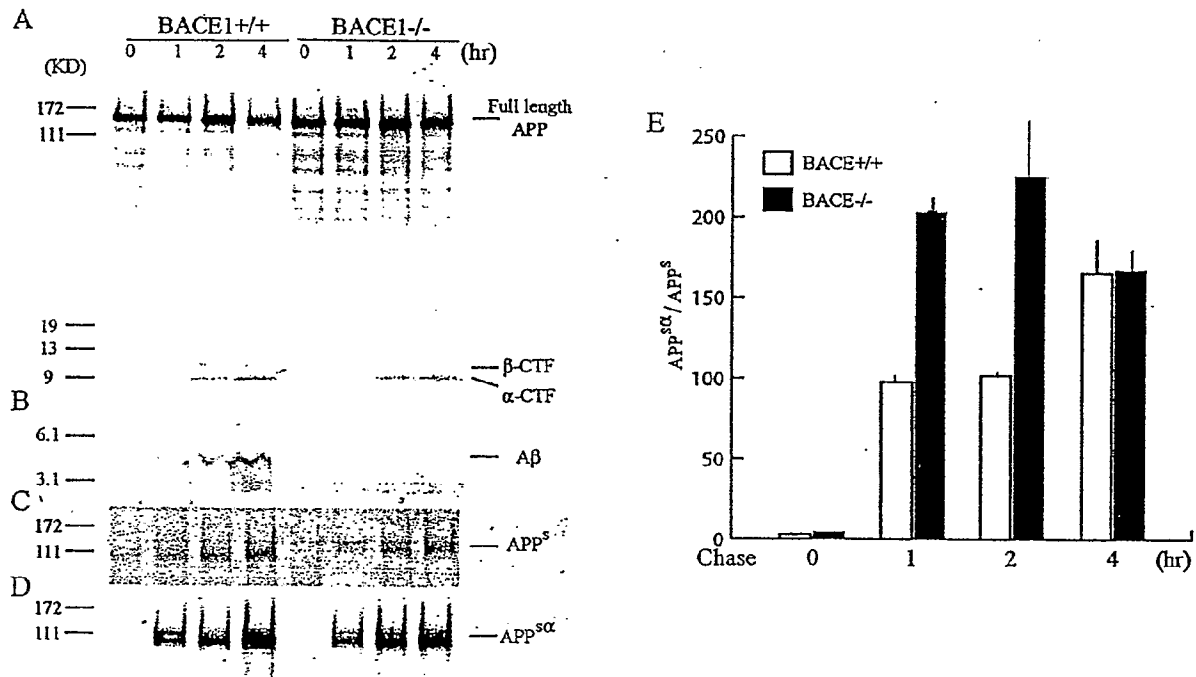


FIGURE 3

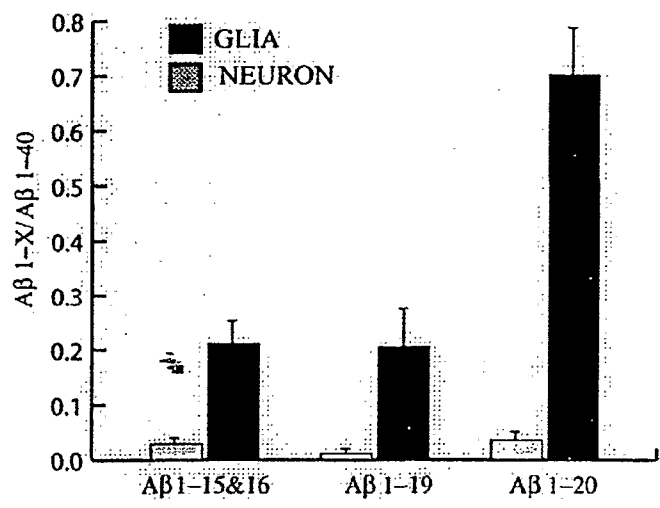


FIGURE 4

## SEQUENCE LISTING

<110> THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE  
 WONG, Philip  
 CAI, Huaibin  
 PRICE, Donald

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 AND METHODS OF USE THEREOF

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